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FURTHER STUDIES ON THE PURIFICATION AND PROPERTIES OF A VIRUS CAUSING TOBACCO NECROSIS.

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In the course of a preliminary survey of some of the viruses causing tobacco necrosis, we (Bawden and Pirie, 1942) found that one culture was sharply differentiated from the others by its small sedimentation constant, its failure to crystallize when precipitated with ammonium sulphate, and by the ease with which it lost infectivity. This Rothamsted culture was derived by bulk transfer from one of the sources of virus distinguished serologically by Bawden (1941), and it is serologically related to one of the components of the mixture of viruses purified by Pirie, Smith, Spooner and McClement (1938). We have now studied a derivative of this culture in more detail and can give a fuller account of its properties. Preparations have still not crystallized when salted out with ammonium sulphate, but when sufficiently concentrated they crystallize readily in the absence of salts. Because of the ease with which this tobacco necrosis virus loses infectivity, the relationship between the crystalline products and the virus as it occurs in the plant is even more uncertain than with the other viruses that we have studied. Using methods that largely destroy infectivity, fully crystalline and apparently homogeneous products are easily prepared. Such products seem to be antigenically identical with infective virus, and antisera prepared against them specifically neutralize infectivity (Kassanis, 1943). Loss of infectivity without loss of serological activity can also be induced with other crystalline viruses that we have studied, and such non-infective preparations crystallize in the same manner as infective preparations. Thus, by analogy with these other viruses, it is likely that infective virus of this Rothamsted culture would crystallize in the same manner as our largely inactivated products, but we do not claim that this is definitely established.

MATERIALS AND METHODS.

The virus used in this work has come from single lesion isolates made from the original Rothamsted culture. Six local lesions were taken, ground up separately and inoculated to healthy plants; the sap from these plants was then tested against a range of antisera prepared against different cultures of tobacco necrosis viruses. Four of the isolates reacted only with antiserum to the Rothamsted culture, and so were

presumed to be the same as the main component present in this culture. One of these four was used as the source for the work now to be described. The results of the tests on the other two isolates showed that our old culture contained a mixture of viruses, and that single local lesions may contain more than one virus ; one reacted with antisera to both the Rothamsted and the potato culture, and the other only with antiserum to the potato culture. Natural infections with tobacco necrosis viruses occur fairly commonly, especially in the winter months ; to reduce the chances of perpetuating contaminants, fresh isolations were made at intervals, from single local lesions, of virus which reacted only with antiserum to the Rothamsted culture.

There is no proof that we have worked continuously with the same virus strain, but there is no evidence to the contrary, and at least all are serologically related. The only suggestion we have that our isolates may not be identical with the main component of the old Rothamsted culture is that purified preparations of the isolates have consistently given lower precipitin titres.

The virus has been propagated in tobacco, *Nicotiana tabacum*, var. White Burley, and French bean, *Phaseolus vulgaris*, var. Canadian Wonder. The yield depends on the number of local lesions produced, and to increase this celite or carborundum (400 mesh) was always incorporated in the inocula. Leaves were picked from 5 to 7 days after inoculation, when the lesions were well developed but before the leaves had become dry and brittle.

The distribution of the tobacco necrosis viruses in the leaf tissues is similar to that of tomato bushy stunt virus (Bawden and Pirie, 1944). When the leaves are passed through a meat mincer, about one-half of the virus is extracted in the sap that can be expressed, but the remainder remains attached to the fibrous residues. This can be liberated either by digestion with commercial trypsin, or by grinding the fibre finely in a triple-roller mill and then extracting with water. Such mill extracts are deep green, and contain some virus combined with the chromoprotein in such a manner that it is not precipitated by virus antiserum. The chromoprotein is easily denatured by freezing in conditions that do not inactivate the virus. Such frozen mill extracts are pale yellow, and afford the most favourable starting material for purification.

For sedimenting the virus we have used an air-driven ultracentrifuge of the type described by Masket (1941) ; 40 minutes at 40,000 r.p.m. (90,000 g.) compacts all the virus in the form of a coherent, rigid pellet, which remains in the tube when the supernatant fluid is decanted. Infectivity and serological tests were made by methods previously described (Bawden and Pirie, 1942). All the antisera used were prepared by Dr. A. Kleczkowski.

PURIFICATION OF THE VIRUS.

Rather different methods of purification are needed with different host plants and at different times of the year. Healthy French beans contain a macromolecular protein which sediments and precipitates with ammonium sulphate along with the virus. This is presumably similar to that found by Loring, Osborn and Wyckoff (1938) in other legumes. It precipitates at pH 4·5, and low-speed centrifugation in these conditions is a necessary step in preparing the virus from beans. This leads to some loss of virus on the precipitate, and to some reduction in the infectivity of that remaining in the supernatant fluid. There is less of this interfering protein in the trifoliate leaves than in the first formed bean leaves, and there is less in the mill extract than in the sap. The ratio of virus to other materials in both tobacco and bean extracts is greater in the dull winter months than in better growing conditions, so that less fractionation is needed with preparations made during the winter.

The leaves are minced, as much sap as possible is squeezed out by hand through madapollam and the residue is minced again. If the leaves are so dry that the weight of sap is less than half the original weight of the leaves, the mince is extracted with a volume of water equal to that of the sap. The fibre is then passed through the triple-roller mill, and the resulting smooth paste is thoroughly mixed with four times its weight of water; after a few minutes' soaking the fluid is expressed. Additional virus can be obtained by a second milling and extraction of the fibre. The sap and extracts are mixed, frozen for 10 to 20 hours at — 8° C. and then thawed. After centrifuging, the virus is precipitated from the supernatant fluid by the addition of 280 g. of ammonium sulphate to each litre. The mixture is centrifuged as soon as the ammonium sulphate is dissolved, for a few hours' exposure to the strong salt solution reduces infectivity. This inactivation cannot be avoided by working at low temperatures, for cooling greatly delays the precipitation and so increases the necessary time of exposure. The precipitate is packed tightly by centrifuging for 45 minutes at 3000 r.p.m.; it is drained well before being suspended in a volume of water equal to one-tenth the volume of the original extracts. Any insoluble material is removed by centrifuging at 10,000 r.p.m. If necessary, the virus in the supernatant fluid can be further fractionated and concentrated by another precipitation with ammonium sulphate, but usually it is now in a suitable state for purification by high-speed centrifugation, and it is centrifuged for 40 minutes at 40,000 r.p.m.

If the original extract is rich in virus, so that the ratio of virus to other sedimentable components is high, the pellets look fibrous to the naked eye and, when examined under the microscope, have the serrated crystalline edges shown in Fig. 1. Fig. 2 is a photomicrograph of the middle of such a pellet. The pellets are suspended in water and left for 1 to 2 hours at 0° C. for the virus to dissolve, when they are centrifuged at 10,000 r.p.m. to remove any insoluble material. The supernatant fluid should now be pale yellow, and when ultracentrifuged again should give pellets which appear to be wholly crystalline and which dissolve completely in water after an hour at 0° C. If there is an insoluble residue, it is removed and the ultracentrifugation is repeated until the pellets dissolve completely.

When small volumes of leaf extracts are handled, more infective products can be made if precipitation with ammonium sulphate is omitted, and fractionation of the frozen leaf extracts is done solely by ultracentrifugation. The pellets that separate from either frozen sap or mill extract from severely affected leaves grown in the winter are often crystalline on the first sedimentation, but sometimes they are brown and apparently amorphous. When resuspended in a volume of water equal to one-tenth of the original volume, much of the pellet does not redissolve. The insoluble material is removed by centrifuging at 10,000 r.p.m., and the supernatant fluid is again ultracentrifuged. The pellets are now fully crystalline and dissolve completely in water. As is shown below, however, these properties are not criteria of homogeneity, because preparations can be fractionated further by precipitating part of the material at pH 4·5 and by making use of the different rates at which different components dissolve.

The infectivity of preparations at this stage is variable, but if the purification has been carried through speedily, 1 ml. of inoculum containing from 10^{-7} to 10^{-9} g. of protein is usually sufficient to produce infection in beans, and at a dilution of 10^{-5} between 50 and 100 lesions per leaf are usual. With its antiserum the preparations give a dense granular precipitate and precipitin titres of from 1/200,000 to 1/300,000. It is necessary to check the precipitin tests with control tubes containing saline instead of antiserum, for if the preparations have not been fractionated with acid, especially if made from infected beans, they are liable to precipitate unspecifically during incubation at 50° C.

Yields of Virus.

The susceptibility of both bean and tobacco plants to tobacco necrosis varies greatly with the season. From November to February plants are extremely susceptible, and inoculated leaves become completely necrotic without the addition of any abrasive to the inoculum. Sap and mill extracts from such leaves give precipitin titres up to 1 in 64 with virus antiserum ; at this time the fragile leaves contain only small quantities of other proteins that interfere with purification, and yields of from 100 to 200 mg. of virus are regularly obtained from a litre of extract. In the spring and autumn large numbers of local lesions are obtained only when abrasive is incorporated in the inoculum. However, the virus content of extracts from leaves well covered by lesions is smaller and precipitin titres in excess of 1 in 8 are rare. The better growing conditions also lead to the production of large quantities of soluble and sedimentable proteins, so that purification is more difficult and yields rarely exceed 10 mg. of virus per litre. During the summer susceptibility is even less, and satisfactory preparations have not been made from plants infected between June and September. No great differences have been found between the yields of virus obtained from bean and tobacco plants grown under comparable conditions and bearing comparable numbers of lesions, but tobacco leaves form the better material for use as inoculum. This is probably because bean leaves contain some inhibitor of infectivity, as more lesions are often obtained from bean sap diluted 1 in 50 with water than from undiluted sap.

If the pH of minced bean leaves is raised, by the addition of sodium hydroxide, from its normal value of 5·7–6·0 to 6·5–7·0, the infectivity of extracts is usually increased appreciably. The yield of purified virus, however, is greatly decreased, presumably because much virus is lost during the removal of the other materials extracted from the leaves by this treatment. The quantity of material that sediments on ultracentrifugation but does not resuspend is doubled by such extraction, and the amount of normal component that sediments and resuspends but is insoluble at pH 4·5 may be increased twenty-fold.

PROPERTIES OF THE VIRUS.

Inactivation.

Perhaps the most characteristic property of this tobacco necrosis virus is the ease with which it loses infectivity without losing its serological activity. In necrotic leaves, or in expressed sap, loss of infectivity is rapid ; within a few weeks at 0° C. only a few lesions may be obtained from material that when fresh was giving hundreds of lesions per leaf. Increasing the temperature increases the rate of inactivation, and at temperatures above 60° C. loss of infectivity with sap or with purified preparations is almost complete within a few minutes, though for loss of serological activity 10 minutes' heating at 90° is necessary. The loss of infectivity is least rapid in salt-free, neutral solutions of the purified virus. We have not studied in any detail the many treatments that lead to loss of infectivity with this culture of tobacco necrosis virus, but we have made some experiments on conditions that do not affect other tobacco necrosis viruses or tomato bushy stunt virus. The most striking of these are exposure to acid and salts. Exposure for 10–20 hours to values between pH 4 and 4·5 leads to a reduction of over 90 per cent. in the infectivity, and we have used this acid inactivation as a criterion to determine whether our products have become contaminated with tobacco necrosis viruses other than the Rothamsted culture. Table I shows the effect of exposure to M/100 acetate buffer at pH 4·4 and 4·1 on three different preparations ; short exposures have little effect, so that by working quickly, the acid-precipitable material in partially purified preparations can be removed without reducing

TABLE I.—*The Effect of Exposure to Slightly Acid Conditions on Infectivity.*

Treatment at 18° C.	Infectivity. Average number of lesions per leaf at 1 : 20,000.	1 : 200,000.
Control	150	51
1 hour at pH 4·4	95	9
17 hours at pH 4·4	14	4
Control	127	41
24 hours at pH 4·1	9	1
Control	123	23
24 hours at pH 4·1	1	0

the infectivity greatly. Loss of infectivity as a result of exposure to neutral salt solutions is less reproducible, for in different experiments exposure to the same conditions has given varying results. We have not compared a large range of different salts, but sodium phosphate causes more rapid inactivation than either sodium chloride or ammonium sulphate. Table II shows the effect of exposing the purified virus to phosphate and sodium chloride for various lengths of time.

TABLE II.—*The Effect of Exposure to Salts on Infectivity.*

Treatment at 18° C.	Infectivity. Average number of lesions per leaf at 1 : 20,000.	1 : 200,000.
Control	162	53
0·065 M phosphate for 1 minute	186	63
,, ,, 210 minutes	71	9
,, ,, 73 hours	6	1
0·26 M NaCl for 73 hours	88	29

Even after ageing for some months, or after prolonged exposure to acid or phosphate, purified preparations still retain 1/1000 or 1/10,000 of their original infectivity. When used as inoculum at 1 mg./ml. they may give an average of three or four lesions per bean leaf. We have on several occasions propagated virus from such lesions to see whether this residual infective virus was a contaminant, but it has always given us preparations indistinguishable from the main Rothamsted culture.

Crystallization.

Preparations of the Rothamsted culture made by the methods described give colourless solutions that are rather clearer than similar solutions of other tobacco necrosis viruses or tomato bushy stunt virus. When centrifuged at 40,000 r.p.m. for one hour in the pH range 5–7 all the virus is compacted into a pellet that is apparently uniform and wholly crystalline. Outside this pH range the pellet is incompletely crystalline, and all the virus is not compacted into the pellet, but some remains in a dense layer of fluid overlying the pellet. It seems that the virus has only a limited solubility in water, for the simplest interpretation of the crystallinity of the pellets is that the concentration of the dense layer of independently sedimenting particles, which is the first result of ultracentrifuging, exceeds this solubility so that the virus

crystallizes as it collects. When partially purified preparations are ultracentrifuged the contaminated pellets are incompletely crystalline, and crystals can often be seen to grow after the tubes are removed from the rotor.

All the other viruses we have studied have given on ultracentrifugation clear jellies, either isotropic or birefringent, which disperse in a few minutes when stirred with water. The crystalline pellets from the Rothamsted culture may remain incompletely dissolved in water at pH 5·5–6·5 for some hours at room temperature. At 0° C. they dissolve more rapidly than at room temperature, and there is more rapid solution in the presence of traces of salt or if the pH is raised to 7·5. Fresh preparations are demonstrably inhomogeneous, for pellets that look fully crystalline contain components that dissolve at different rates and have different infectivities. This can be shown by suspending the crystalline pellet in water, and centrifuging immediately at 3,000 r.p.m. to deposit the undissolved fraction. When the two fractions are compared they give the same serological titre, but the more quickly dissolving fraction has the greater infectivity (Table III). The results given in the accompanying paper (Ogston, 1945)

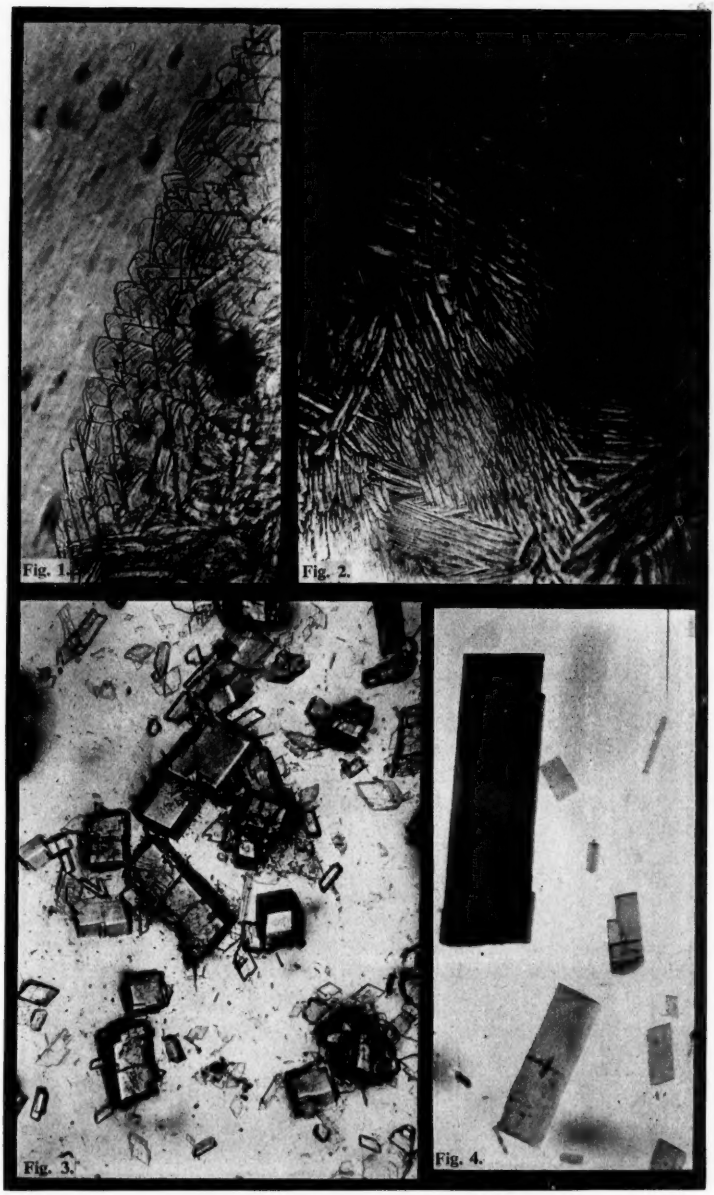
TABLE III.—*Infectivity of Successive Extracts of Crystalline Pellets.*

Extract.	Weight. (mg.)	Average lesions per leaf at 10^{-4}.	Average lesions per leaf at 10^{-5}.
First	11	430	230
Second	5·3	240	63
Third	1·9	89	17
Final	0·3	30	7
First	5·5	170	87
Final	1·7	84	12
First	10	188	34
Final	3	92	7

The results of experiments with three different preparations are given. Four successive extracts of the crystalline pellet were made with the first preparation; the first extract was made by stirring the pellet with water and centrifuging immediately at 3000 r.p.m.; the second and third were each extracted 30 minutes before centrifuging, and the final extract was left for 24 hours. Only two extracts were made of the second and third preparations; the first was made by stirring the pellet in water and centrifuging immediately, and the final was left for 24 hours.

show that both fractions are centrifugally homogeneous, and have the same sedimentation constant. With fresh preparations the rapidly dissolving fraction is the main component, though this itself is not homogeneous, for if it is ultracentrifuged it can usually again be fractionated by washing the crystalline pellet. Parallel with the fall in infectivity as a result of ageing or exposure to pH values between 4 and 5, the proportion of the pellet that dissolves rapidly also falls, and in preparations that are almost non-infective little or nothing goes into solution during such a quick extraction.

The decrease in solubility as a result of ageing probably explains the second type of crystallization given by the Rothamsted culture, for this occurs in stored solutions made by dissolving the crystalline pellets. If such solutions are more concentrated than 10 g./l., after some days or weeks at 0° C. they deposit crystals on the walls of the vessel. Fig. 3 shows their appearance soon after crystallization has begun, but growth continues for several months, and laths up to $1 \times 2 \times 6$ mm. have been produced (Fig. 4). By this time the preparation will have lost substantially all its infectivity. Crowfoot and Schmidt (1945) state that these crystals are of two kinds,



Crystals of the Rothamsted Culture of Tobacco Necrosis Virus.

FIG. 1.—The serrated edge of a crystalline pellet produced by ultracentrifugation. × 45.
FIG. 2.—Centre of the pellet shown in fig. 1. × 45.
FIG. 3.—Crystals separating from a salt-free solution after standing for some days. × 45.
FIG. 4.—Large laths produced after crystal growth has continued for three months. × 9.

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thick triclinic prisms and thin hexagonal or pseudo-hexagonal plates, and they have made X-ray crystallographic measurements on them. The part of a preparation that separates as crystals does not differ in any of the properties we have studied from that remaining in solution. Solutions of the crystals and their mother liquors give the same serological titre. When ultracentrifuged, they both give crystalline pellets indistinguishable in appearance from those given by freshly made preparations, though they dissolve much more slowly than these.

Repeated attempts have been made to get crystals by slow precipitation with ammonium sulphate, using the technique that was successful with the other cultures of tobacco necrosis virus, but all have failed. The potato culture resembles the Rothamsted culture in that it also crystallizes from salt-free solutions (Bawden and Pirie, 1942). If the lozenge-shaped crystals produced by precipitating the potato culture with ammonium sulphate are dissolved to give a solution containing more than 5 g. per l., crystals begin to separate after some time at pH 4·5, which resemble those produced by ammonium sulphate precipitation. Crystallization of the potato culture, however, is not associated with loss of infectivity, and there is no difference between the infectivity of the fraction of a preparation that crystallizes in this way and that of the fraction remaining in solution.

When adjusted to pH 4·5 most preparations have given a precipitate, which with those made from infected beans has sometimes been as much as 30 per cent. of the whole preparation. We do not regard precipitability from dilute salt solutions at pH 4·5 as a property of the virus, however, because some preparations made from tobacco leaves, which were as infective as any we have handled, were completely soluble at this pH. If the precipitate and supernatant fluid are separated and neutralized quickly and their serological activities and infectivities compared, the precipitated material gives a smaller serological titre than the soluble part, though it often is more infective. Sometimes indeed nearly the whole of the infectivity is associated with the precipitate, though more than two-thirds of the material precipitating with virus antiserum remains soluble. Similar precipitates were encountered when preparations of tobacco cultures I and II (Bawden and Pirie, 1942) were brought to pH 4·5, and we suggested that the virus was being absorbed during the precipitation of some other leaf component. To explain the behaviour of the Rothamsted culture on this basis involves the assumption that infective virus particles are more readily absorbed by such leaf components than non-infective particles. This seems probable, but there is no positive evidence for it ; when sedimentable protein from healthy bean leaves was precipitated at pH 4·5 in the presence of a virus preparation which was soluble at this pH, there was no such preferential absorption.

Chemical Composition.

The carbohydrate and phosphorus contents of purified preparations of the Rothamsted culture fall within the ranges 7·0–8·5 per cent and 1·7–2·0 per cent respectively, i.e. similar to those described for other viruses causing tobacco necrosis (Pirie Smith, Spooner and McClement, 1938 ; Bawden and Pirie, 1942). No consistent differences have been found between the fractions with different rates of solubility into which a preparation can be separated, or between freshly made, infective preparations and old, non-infective preparations. The ratio of carbohydrate to phosphorus is that characteristic of a nucleic acid, and material with the physical properties of nucleic acid can be separated from the protein by exposure to dilute alkali. After precipitating the denatured protein with 5 per cent ammonium sulphate solution, nucleic acid can be precipitated from the fluid by the addition of strong acid. Colour

reactions show that the nucleic acid contains a pentose. The Rothamsted culture of tobacco necrosis virus, like other plant viruses previously studied, is, therefore, a nucleoprotein of the ribose type, but we have not demonstrated that the sugar is actually ribose.

DISCUSSION.

In this paper the crystalline product we have isolated from plants infected with the Rothamsted culture of tobacco necrosis virus has been referred to as virus. This has been done mainly because of analogies with other viruses with which we have worked, for there is, in fact, no positive evidence that any of the properties of our product are those of the virus itself. Indeed, there is positive evidence that our infective preparations are heterogeneous, and no conclusive evidence that infective virus can be obtained in crystalline form. However, the simplest interpretation of our results is that the virus particles readily lose infectivity without suffering gross changes, and that in the infective and non-infective states they have the same size, crystal forms and serological activity. Other viruses can be made to undergo such losses of infectivity and, in the absence of contrary evidence, it is reasonable to think that treatments which produce loss of infectivity in the Rothamsted culture, but which have no effect on other tobacco necrosis viruses or on tomato bushy stunt virus, cause this type of inactivation. On this assumption, our purified preparations would invariably contain a mixture of infective and non-infective particles, and the variation in infectivity of different preparations is simply a reflection of the different proportions in which infective virus and the modified, non-infective derivative are present.

There is one important difference between the behaviour of the Rothamsted culture and the other viruses. When crystals of partially inactivated preparations of tomato bushy stunt virus or the potato culture of tobacco necrosis virus are dissolved, there is no difference between the infectivity of the material that dissolves rapidly and that which dissolves slowly, whereas the results given in this paper show that when crystalline ultracentrifuge pellets of the Rothamsted culture are suspended in water the infective material dissolves more readily than the non-infective. Thus if these pellets are wholly crystalline, as their appearance suggests, then it seems that loss of infectivity has no effect on crystal form or serological activity, but reduces the rate of solution. The appearance of crystallinity, however, is not trustworthy as an index of homogeneity, for if purified preparations of the potato or tobacco II cultures of tobacco necrosis are added to purified preparations of the Rothamsted culture in the ratio of 1 to 4 by weight, ultracentrifugation gives an apparently homogeneous and wholly crystalline pellet, although neither of these other viruses crystallizes in these conditions, and one-fifth of the "crystalline" pellet is a non-crystalline, invisible gum. An obvious alternative explanation of our results, therefore, is that the crystalline material is wholly non-infective, derived from virus by changes insufficient to affect most properties, and that infective virus is not crystallizable, but occurs in the pellets around the crystals as an invisible gum, which dissolves rapidly when dispersed in water. To explain the further crystallization of such solutions when again ultracentrifuged, it is necessary to postulate incomplete fractionation or subsequent inactivation leading to crystallizable material.

Loss of infectivity during the course of purification is not a necessary preliminary to crystallization. In the winter months, when conditions are such that frozen leaf extracts contain little sedimentable material except virus, crystalline pellets are often obtained at the first centrifugation, and if these are suspended in their own supernatant fluids they are as infective as the original extracts. Thus if infective virus does

not itself crystallize, then it seems that freshly-expressed extracts from infected plants must already contain much non-infective virus.

A third interpretation of our results is that the virus is only a minor component of our purified preparations, and that our crystalline material is merely a specific disease product. A similar suggestion could be made for all the other purified virus preparations, but it necessitates the introduction of extra hypotheses with no obvious advantage. With the Rothamsted culture it would also be necessary to postulate that the rate of solution of the specific disease product alters as the virus loses its infectivity. The fewest hypotheses are needed if we assume that the virus readily changes into a non-infective derivative without altering any of its other properties that have been tested except rate of solution. On this assumption, this plant virus seems to have smaller particles than any other previously studied. The X-ray crystallographic measurements suggest that the particles are spherical and have a minimum weight equivalent to a molecular weight of 1,600,000 (Crowfoot and Schmidt, 1945), which agrees well with the figure of 1,850,000 calculated from the sedimentation constant, assuming that the particles are spherical, unsolvated and obey Stokes' law.

SUMMARY.

A nucleoprotein that is not present in the leaves of healthy plants has been isolated from bean and tobacco leaves infected with the Rothamsted culture of tobacco necrosis virus. This has not crystallized when precipitated with salt, but it crystallizes slowly from concentrated salt-free solutions or during sedimentation by ultra-centrifugation. It has a sedimentation constant of 49S, smaller than that of other preparations of plant viruses previously studied. The Rothamsted culture of tobacco necrosis virus readily loses infectivity, and the relationship between the crystallizable protein and the virus is uncertain. It is most likely that much of the protein is a non-infective derivative of the virus having many physical, chemical and serological properties in common with it.

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ULTRACENTRIFUGAL EXAMINATION OF A VIRUS CAUSING TOBACCO NECROSIS.

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THREE samples of tobacco necrosis virus, prepared by Bawden and Pirie (1945), were examined in a Svedberg oil-turbine ultracentrifuge. The samples were :

595L : The whole (preparative) ultracentrifuge pellet redissolved.

845H : The more rapidly dissolving fraction of a pellet.

845G : The corresponding, more slowly dissolving, residue.

Sedimentation was observed by the "diagonal schlieren" method (Philpot, 1938) ; the speed of rotation was 300 to 320 rev./sec. ; the solutions of virus in 0·1

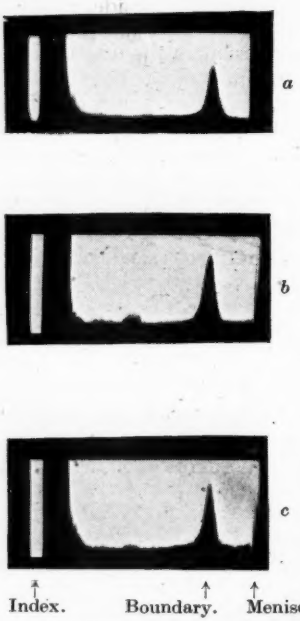

FIG. 1.—Diagonal schlieren diagrams of (*a*) 595L, (*b*) 845H, (*c*) 845G after 50 min. at 315–320 rev./sec. Optical sensitivities are : (*a*) 48°, (*b*) 62°, (*c*) 55°. The second hump appearing in (*b*) and (*c*) is due to a base-line irregularity, not to a sedimenting component.

M NaCl and 0·01 M acetate buffer of pH 4·7 were contained in a 12 mm. cell. The amounts of refracting material appearing in the boundaries were compared with the total refractive increment of the virus in solution by a modification of the method of Philpot (1939) ; instead of measuring the areas above the baseline in the diagram on a direct positive print, they were measured on a tracing of an enlarged projection of the

original negative. This gives somewhat improved accuracy by making possible a more accurate fitting of the baseline and the measurement (by planimeter) of a larger area. The method is still not as accurate as could be desired. The specific refractive increment of the virus in water was found to be 0·00156 for light of wave-length 560–600 mμ.

The results are given in Table I and in Fig. 1.

TABLE I.

Preparation.	Concentration. g./litre.	S_{20} (corr.) $\times 10^{13}$.	Fraction of total refraction found in boundary.
595L	2·5	49·9	0·82
845H	2·55	48·5	0·90
845G	1·79	49·7	0·91

Each preparation contained only a single visible component, apparently homogeneous; the recovery in the boundary of refractive material is complete within the accuracy of the method for 845H and G, considering their low concentrations and the high optical sensitivity required to obtain the diagrams; 595L may contain a little refracting material which does not appear in the boundary, but this is by no means certain.

The sedimentation constants do not differ significantly; in none of the preparations was there any sign of the component having $S_{20} = 240 \times 10^{13}$ observed in preparation 488V (Ogston, 1942). The ultracentrifuge therefore reveals no difference between the fractions of the pellets which show different rates of solution.

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AN ANTI-GRAMICIDIN IMMUNE RABBIT SERUM.

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FOR experiments designed to determine the sequence of amino-acid residues in the polypeptide-like substance gramicidin, it was desirable to prepare antisera specific for gramicidin in the hope that specific inhibition effects with relevant synthetic peptides (Landsteiner and van der Scheer, 1932, 1934, 1939; Landsteiner, 1942) may give a clue to sequences in the molecule not readily capable of eludication purely by the study of partial hydrolysis products (Synge, 1943, 1944).

Attempts by the usual methods to immunize rabbits with gramicidin were unsuccessful, but antisera which precipitated with gramicidin were obtained by injecting

gramicidin together with the conjugated protein derived from the O-antigenic complex of *Bact. shigae* (Morgan, 1937 ; Morgan and Partridge, 1939, 1940, 1941), a procedure which has already proved effective in the preparation of antisera specific for various polysaccharides and related substances (Partridge and Morgan, 1940, 1942 ; Morgan, 1943, 1944*a* ; Freeman, 1943 ; Morgan and Watkins, 1944).

In the present paper the preparation and some of the properties of antisera capable of reacting with gramicidin are described.

EXPERIMENTAL.

Gramicidin.

The recrystallized specimens W2 and R3 from two different batches of gramicidin were employed. Their preparation and properties are described by Synge (1944).

Conjugated protein.

Two separate preparations of the O-somatic antigen of *Bact. shigae* were broken down into their constituent molecules by hydrolysis with 0·1 N acetic acid at 100° for 4–5 hours, and the conjugated protein component isolated and purified according to the procedure described by Morgan and Partridge (1940, 1941). The material was homogeneous when examined at pH 8·0 in the Tiselius electrophoresis apparatus. The two substances contained 10·9 per cent N and 1·06 per cent P, all of which was organically bound, and 11·2 per cent N and 1·05 per cent P respectively. The materials showed a laevo rotation $[\alpha]_{5461}$ — 50° and — 48° (C, 0·5 per cent in 0·05 N NaOH).

The antigenic complex.

Gramicidin (10 mg.) was dissolved in 1·4 ml. ethanol and to the solution was added 7·3 ml. distilled water. Conjugated protein (1 mg.) was dissolved in 2·0 ml. 0·05 N NaOH and the two solutions were mixed. The opalescence of the alkaline mixture was markedly less than that of the gramicidin solution alone. The mixture was allowed to remain alkaline for 5–10 minutes at room temperature, and was then acidified (pH 4·7) with 0·7 ml. 0·3 N acetic acid. Precipitation occurred. The resulting suspension stored at 0–2° C. was employed for injection.

Immunization procedure.

Each course of immunization consisted of 3 intravenous injections at 3–4 days' interval ; the sera were collected from the rabbits 10–14 days after the last injection of the course.

In the preliminary experiments 1 ml. injections of 1 mg. gramicidin were given to 5 rabbits ; the solution for injection was made by diluting a stock alcoholic solution of gramicidin in water or 5 per cent w/v aqueous glucose solution. In view of Landsteiner's (1942) successful preparation of antisera to silk, a protein-like substance which resembles gramicidin in its high content of monoamino monocarboxylic residues, we hoped that the administration of gramicidin similarly adsorbed on carbon would be effective in inducing the formation of antisera that would react with gramicidin. Three rabbits therefore received the same dose (1 mg. gramicidin) to which 15 mg. of blood charcoal had been added. The charcoal rapidly adsorbed the gramicidin, and the previously opalescent solution became quite clear. Three further rabbits were given intraperitoneally doses of the same suspension of gramicidin adsorbed on carbon

but the doses were larger, and in all 3 doses each of 5 mg. and 1 dose of 10 mg. were given at weekly intervals.

Sera obtained after two intravenous courses of gramicidin (alone or adsorbed on carbon) and after 1 intraperitoneal course of gramicidin adsorbed on carbon failed to yield a precipitate when mixed with several different concentrations of gramicidin.

A group of rabbits also received a series of 6 or 9 doses, each of 1 mg. of gramicidin after it had been mixed with an alkaline solution of conjugated protein and acidified as described above. Each animal in the group gave rise to an immune serum which formed a precipitate when mixed with a solution of gramicidin. Gramicidin preparations W2 and R3 were both employed as antigens after being combined with conjugated protein.

A control group of rabbits received exactly parallel injections of gramicidin alone, but which had been treated in the same way with ethanol, NaOH and acetic acid. The appearances noted in preparing this mixture differed in no way from those when the conjugated protein was present, and it failed to induce the formation of anti-gramicidin precipitins. A number of rabbits were also given injections of conjugated protein alone; the resulting sera showed a weak precipitation reaction with the homologous antigen only.

If in a good state of health and nutrition none of the rabbits showed any adverse reactions to these injections. The material for injection was made up with sterile precautions and stored at 0–2°. Serum samples collected during the immunization were stored at — 20° without addition of antiseptic.

Precipitation Reaction.

Methods.

The best conditions found for observing the precipitation of gramicidin by antisera were the following and they were employed throughout the work. Gramicidin (0·5 per cent w/v) in ethanol was diluted to 10 times its volume with distilled water, and 0·1 ml. of the resulting freshly prepared colloidal suspension or of dilutions of it in distilled water was mixed with 0·1 ml. of serum. The mixture was incubated for 2 hr. at 37° and kept overnight at 0°. Longer storage at 0° or the use of aged aqueous gramicidin suspensions increased the extent of non-specific precipitation of gramicidin, which, under the conditions recommended, was slight. Gramicidin in the highest final concentration employed (1 : 4000) conferred a marked opalescence on normal serum, but there was little or no precipitation. With the immune sera finely divided muddy precipitates were formed, and there was little or no opalescence in the supernatant. The precipitates settled very slowly after being shaken up. Some of the more powerful immune sera gave a precipitate after the incubation at 37° and before cooling to 0°.

Results.

After the first course of injections of gramicidin with conjugated protein the precipitation reaction with gramicidin was just detectable. After two immediately consecutive courses 7 out of 9 rabbits gave sera reacting strongly, and after 3 consecutive courses all rabbits gave the maximum reaction observed in the course of this work. Representative results are shown in Table I.

Gramicidin R3 was employed in all the precipitation tests. The fact that it reacted equally with antisera prepared against specimens R3 and W2 is further evidence for the identity of the gramicidin in the two batches (Synge, 1944).

TABLE I.—*Precipitation Reactions of Sera with Gramicidin.*

No. of rabbit.	Material injected.	Specimen of gramicidin injected.	No. of courses.	Final concentration of gramicidin in test. 1 : 4,000.	1 : 16,000.	1 : 64,000.	1 : 256,000.	(Control without gramicidin.)
Average normal serum.	—	—	—	1	0	0	0	0
778	G + P	R3	3	4	2	2	1	0
779	G + P	R3	3	3	2	1	0	0
781	G + P	R3	3	4	3	1	0	0
843	G + P	W2	3	4	2	1	–	0
844	G + P	W2	3	4	3	1	–	0
846	G + P	W2	3	4	3	1	–	0
836	G	W2	3	1	1	0	–	0
838	G	W2	3	1	0	0	–	0
842	G	W2	1	0	0	0	–	0
Pooled	P	–	2–4	0	0	0	–	0
778*	G + P	R3	4	1	0	0	0	0
779*	G + P	R3	4	1	0	0	0	0

G = Gramicidin. P = Conjugated protein.

4, very heavy precipitation. 3, heavy precipitation. 2, definite precipitation.
1, trace of precipitation. 0, no precipitation.

* Serum sample obtained after the immune animal had received an intravenous injection of 10 mg. of gramicidin.

Acute Toxicity of Gramicidin for Normal and Immunized Rabbits.

Suspensions of gramicidin obtained by diluting a 5 per cent w/v solution in ethanol to 10 volumes with distilled water were administered intravenously. The results are given in Table II. The rabbits weighed 2–3 kg.

The toxicity data are consistent with those obtained for intravenous administration by other authors (Hotchkiss, 1944). The impression was gained, in agreement with

TABLE II.—*Acute Toxicity of Gramicidin for Normal and Immune Rabbits.*

Rabbit No.	Material injected.	No. of courses.	Dose of Gramicidin R3 in Toxicity Expt. (mg.).	Experiment No.	Time to death.
630	Normal	–	10	1	5 min.
596	,,	–	5		4 days
610	,,	–	5	2	1 day
624	,,	–	5		1 ,,
480	G	1	5		1 ,,
547	G	1	5		1 ,,
549	G	1	5		1 ,,
573	G	1	5		1 ,,
767	Normal	–	10	3	1½ days
769	,,	–	10		3 ,,
778	G + P	4	10		Survived
779	G + P	4	10		,,
781	G + P	4	10		1 day
843	G + P	3	10	4	1½ days
844	G + P	3	10		2 days
846	G + P	3	10		1 day
836	G	3	10		½ ,,
838	G	3	10		1 ,,
842	G	3	10		¾ ,,

G = Gramicidin. P = Conjugated protein.

Hotchkiss' view, that the speed and nature of the toxic effect depends considerably on the colloidal state of the gramicidin suspensions employed for injection ; in the present work, uncontrolled factors caused this to vary from experiment to experiment.

None of the rabbits that died took food later than 12 hr. after injection. At autopsy petechial haemorrhages of lungs and/or kidneys and haemoglobinuria were present in most, but not all animals.

In Experiment 3 the two animals which survived were obviously ill, and did not take food for 3 days, after which time recovery was rapid ; they were killed a fortnight later, in apparent good health. At this time their sera (Table I, 778,* 779*), had lost the power of yielding a precipitate on the addition of gramicidin.

It is concluded from these experiments that the animals immunized with gramicidin together with conjugated protein and possessing serum precipitins that react with gramicidin are very slightly less susceptible to the toxic action of gramicidin administered intravenously than are normal rabbits and rabbits that have received injections of gramicidin alone.

Effect of Antisera on the Antibacterial Action of Gramicidin.

Experiments were conducted to ascertain whether sera precipitating gramicidin also inhibited its antibacterial action. Serial dilutions of gramicidin in serum diluted 1 : 5 with water were tested for their power to inhibit growth of a susceptible strain of *Staphylococcus aureus* by the slide-cell technique of Fleming (1943). With three of the most potent precipitating sera and three normal sera no consistent differences could be detected. In each instance bacteriostasis occurred at gramicidin concentrations of 6–20 μg./ml.

Effect of Antisera on the Haemolytic Action of Gramicidin.

Experiments were conducted as described by Mann, Heilman and Herrell (1943). Under sterile conditions to 0·1 ml. of a 2 per cent. washed suspension of rabbit red cells in normal saline was added 0·1 ml. of the serum under test and 0·02 ml. of serial aqueous dilutions of gramicidin. The mixtures were incubated at 37° and observed hourly. Four serum specimens (A, B, C and D) were tested in the same way :

A	pooled precipitating sera of rabbits	778, 779, 781	(G + P)
B	,, ,, ,, ,,	843, 844, 846	(G + P)
C	,, non-precipitating sera of rabbits	836, 838, 842	(G)
D	,, ,, ,, ,,		(P)

Haemolysis was progressive, and there was no significant difference in the results with the different sera. The range of variation observed is shown in Table III.

TABLE III.—*Course of Haemolysis of Rabbit Cells in* (1 : 2) *Diluted Sera by Gramicidin.*

Time of incubation (hr.)	Degree of haemolysis observed with different sera. Final concentration of gramicidin in mixture (μg./ml.)				
	50.	12·5	3·1.	0·8.	0 (Control without gramicidin.)
1	¼–¾	0–¼	0	0	0
2	C	¾–C	tr.–½	0	0
3	C	C	¾–C	0–½	0
4	C	C	C	0–C	0
5	C	C	C	tr.–C	0
6	C	C	C	¼–C	0

0 = No haemolysis. C = Complete haemolysis.

Haemolysis was somewhat more rapid than that observed with the sheep-cells in 1 : 2 horse serum by Mann, Heilman and Herrell (1943).

DISCUSSION.

The results of the experiments which have been described show that the injection into rabbits of an artificial complex of gramicidin and conjugated protein gives rise to an immune-body which precipitates with gramicidin.

The conversion into antigens of non-antigenic or weakly antigenic polysaccharides by combining them with the conjugated protein of the O-somatic antigen of *Bact. shigae* has already been described (Morgan, 1944*a* and *b*). All of the substances studied previously, however, are predominantly polysaccharide in nature. Gramicidin, although possessing some free –OH groups (Hotchkiss, 1941 ; Synge, 1945) is otherwise of very different chemical character. The present results therefore suggest that the property of the conjugated protein of facilitating the production of antisera reacting with substances themselves poorly or not antigenic may be widely applicable. In this particular instance the activity cannot be attributed simply to the formation of a particulate antigen, since the administration of gramicidin adsorbed on carbon was ineffective. Nor do the incomplete data at present available suggest that the properties of forming an antigenic complex with non-antigenic substances is common to many proteins other than the conjugated protein components of the O-somatic antigens of the Salmonella group of organisms. Control experiments made by injection of gramicidin alone show clearly that by itself gramicidin is not an antigen, and this is confirmed by the observation that the precipitation reaction of the serum of rabbits immunized with the complex antigen was eliminated after injection of a relatively massive dose of gramicidin alone (Table I, Rabbits 778, 779). The possibility of cross reaction between gramicidin and antibody formed in response to conjugated protein alone is excluded by the negative results obtained in precipitation tests between gramicidin and an anticonjugated protein immune serum.

The effect of antisera capable of precipitating gramicidin on the power of gramicidin to inhibit the growth of staphylococci and to effect lysis of red blood corpuscles has been investigated by *in vivo* tests. In neither instance was any effect detected. Under the conditions of the experiments, the gramicidin was present in concentrations less than that necessary for the formation of a precipitate with the antiserum ; it may therefore be accepted that the gramicidin was saturated with antibody at most or all sites capable of forming a specific combination with the antibody, and that the immunologically active receptor groups are different from, and independent of, the groups responsible for the antibacterial and haemolytic activity of gramicidin. There is evidence from the results of bacteriostatic and haemolytic experiments with cephalin that the bacteriostatic and haemolytic actions of gramicidin may be attributed to the same fundamental mode of action (Hotchkiss, 1944).

The slight diminution observed in the toxicity of gramicidin for rabbits possessing precipitins against it is no more than could be attributed to the " physical " effect of the antibody in delaying the escape of gramicidin from the circulation. Furthermore, since in the presence of excess antibody the haemolytic action of gramicidin *in vitro* seems in no way diminished, this definite although slight diminution in toxicity for immunized animals suggests that the toxic effects of intravenous administration cannot be attributed entirely to the haemolytic action of gramicidin on the red blood corpuscles.

Biologically active substances in their reaction with specific antisera vary in the degree to which their activities are affected by the state of combination ; for example,

diphtheria antitoxin (horse serum) is precipitated by an antiserum to horse pseudoglobulin and the precipitate so formed combines with diphtheria toxin (Smith and Marrack, 1930; Treffers, 1944). The experiments described in the present paper similarly show that the antibacterial and haemolytic activity of gramicidin is not significantly diminished by the combination of the gramicidin with antibody which has been engendered as a result of immunization with a gramicidin—conjugated protein antigen.

SUMMARY.

1. The results of immunization experiments are given in which rabbits were inoculated with gramicidin, with a conjugated protein, and with an artificial complex built up from gramicidin and conjugated protein.

2. Gramicidin failed to induce the formation of demonstrable immune-body. The conjugated protein engendered homologous precipitins, but none reactive with gramicidin.

3. The artificial complex induced the formation of precipitating sera which reacted strongly with gramicidin. Gramicidin adsorbed on charcoal was not antigenic.

4. Gramicidin in the presence of excess homologous precipitin retains its bacteriostatic and haemolytic properties unimpaired.

5. Rabbits possessing potent gramicidin precipitins are slightly more resistant to the toxic action of an intravenous dose of gramicidin than are normal animals.

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THE SEPARATION AND PROPERTIES OF TOBACCO MOSAIC VIRUS IN DIFFERENT STATES OF AGGREGATION.

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THE problem of the size and shape of the particles of tobacco mosaic virus has received much attention for many years, and the conclusions reached have been about as numerous and varied as the workers engaged and the methods employed. For long it was tacitly assumed that the particles were incompressible spheres and calculations of size were made on this basis, although Takahashi and Rawlins suggested in 1933 that they might be rod-shaped. Studies of purified virus preparations within the last nine years have proved that the virus can occur in the form of greatly elongated particles. We contributed some of the evidence for this, but we also suggested that these particles may be artifacts, produced during the course of purification by the linear aggregation of smaller particles, for the purified virus filtered less readily, was less infectious, and showed more anisotropy of flow than virus in clarified sap (Bawden, Bernal and Fankuchen, 1936; Bawden and Pirie, 1937*a*). Infective sap contained some rod-like particles, for it showed some anisotropy of flow, but there was no evidence to show whether all the virus present was of a uniform size and shape. We have urged the need for caution in translating physical data on tobacco mosaic virus into "molecular" weights and sizes, for the physical properties are so anomalous that conclusions based on them are suspect. Even if the underlying theory were unequivocal, in a system that aggregates so readily there is a danger that the size calculated might be neither that of the smallest particle capable of causing infection nor that of the most common particle in untreated material.

Loring, Lauffer and Stanley (1938) agreed that virus in sap aggregates linearly when precipitated with acid, salts or alcohol, but claimed that homogeneous, unaggregated preparations could be made by ultra-centrifugation of infective sap. At different times various weights and sizes have been assigned to the particles in such preparations, but since 1941 Stanley and Lauffer have postulated a basic particle or molecule about 15mμ. thick and 280mμ. long. They consider that aggregation is restricted to the end-to-end joining together of two or three such fundamental particles.

Evidence from a number of different sources suggests that particles of approximately this size are common in preparations of tobacco mosaic virus, but it seems unjustifiable to assume that these are the equivalent of virus "molecules" when there is nothing to show that they are not simply one of the more stable aggregates. Indeed there are suggestions that smaller particles exist. For example, Frampton (1942) measured the approximate lengths of 159 particles shown in electron micrographs of tobacco mosaic virus published by Stanley and Anderson (1941), and pointed out that many were shorter than the postulated molecules, some being as short as 37mμ. However, this is not conclusive, for drying inactivates the virus and reduces the anisotropy of flow (Bawden and Pirie, 1937*a*); it may therefore be causing breaks in the rods. There is no published evidence to show whether these small fragments are formed by a disruption of the fundamental particle during the drying involved in the

preparation of the electron microscope specimen, or whether they are the true units from which the larger particles are built by aggregation.

In the present paper we describe further experiments showing how readily the properties of tobacco mosaic virus can be altered. Preparations made by the method we have described recently show much less anisotropy of flow than our first preparations (Bawden and Pirie, 1943, 1937*a*). They need to be more concentrated to give a liquid crystalline layer and they give lower serological titres, although they behave qualitatively in precipitin tests in much the same manner. Heating the sap to 70° C. and incubation with trypsin were steps in our first method that were omitted later; the differences arise from these omissions, and by restoring either of these treatments preparations are obtained showing the extreme orientation phenomena. The differences between these preparations made by different methods are insignificant compared with those between such preparations and those that can be made by using carefully chosen starting material. From such material we have prepared virus solutions with physical properties so different from those of our earlier preparations that it now seems probable that the particles responsible for the anisotropy of flow in infective sap are themselves aggregates.

Virus preparations with different physical properties also behave very differently in serological tests. As ordinarily prepared, tobacco mosaic virus gives with its antiserum an open, fluffy type of precipitate, resembling that given by bacterial flagellar antigens. We attributed this to elongation of the particles, for other anisometric viruses behave similarly, whereas viruses with spherical particles give dense, granular precipitates, similar to those given by bacterial somatic antigens (Bawden and Pirie, 1938). We now have further evidence for the view that serological behaviour depends on the shape of the antigen, for preparations of tobacco mosaic virus that show little or no anisotropy of flow resemble somatic rather than flagellar antigens in precipitin tests. Such preparations are readily transformed into ones showing anisotropy of flow and they then behave like flagellar antigens.

MATERIALS AND METHODS.

Most of this work was done with a mild strain of the common tobacco mosaic virus propagated in tobacco, var. White Burley, but essentially similar results were obtained with this and with tomato aucuba mosaic virus propagated in tomato, var. Kondine Red.

In previous work on tobacco mosaic virus the sap that is expressed from leaves after maceration with a pestle in a mortar or by passage through a meat mincer has been used. We have tried many different kinds of leaf extract in experiments preliminary to those now described, and these have shown that tobacco mosaic virus aggregates as a result of short exposures to conditions obtaining in sap from minced whole leaves. The virus isolated from sap may, therefore, be in a different state from that in which it occurs in infected cells. That this aggregation proceeds in expressed sap but not in intact cells need occasion little surprise, for the sap from minced leaves probably has little or nothing in common with any fluid that exists in intact cells. For simplicity, only one procedure for extracting virus in its least aggregated form will be described.

Preparation of Virus-containing Extracts.

Leaves between 2 and 5 in. long are picked about a month after the tobacco plants have been infected, the midribs are cut out and discarded, and the blades are frozen for 5–20 hours at — 10°. The frozen block of leaves is put into 30 times its weight

of water at room temperature and stirred gently till thawed ; as much fluid as possible is pressed out by hand without rubbing or shearing. This brown fluid contains some virus and its properties will be described later. The mass of leaf blades is carefully unravelled, suspended again in 30 times its weight of water and pressed out ; this washing is repeated. Enough of the leaf components that normally lead to aggregation in sap have now been removed for relatively stable extracts of virus to be made. The leaf blades are passed through a domestic meat mincer, the fibre is mixed with a little water, squeezed in a cloth and the residue reminced and re-extracted. The weight of the combined extracts is about three-quarters of the original weight of the leaf blades, i.e. the volume of extract is about equal to the volume of sap that would have been got by direct mincing. The extract is at pH 6·0–6·5 and, after centrifuging for half an hour at 3000 r.p.m., it is clear and pale brown. It will be called " mincer extract." Residual free virus in the mince can be removed by repeatedly suspending the fibre in water and pressing. This washing is generally combined with neutralization for the pH of the mince falls, as in the comparable experiments on the liberation of tomato bushy stunt virus (Bawden and Pirie, 1944), because of the demethylation of pectin (Holden, 1945). Four washes with 60 ml. of water to 10 g. of fibre are sufficient to reduce the virus content of the last wash to one thousandth of that of the first extract after mincing.

The washed fibre is now moistened to get a suitable consistency and passed through the triple-roller mill which we have described previously (Bawden and Pirie, 1944). The smooth paste is suspended in about 5 parts of water ; after a few minutes the fluid is separated either by squeezing through madapollam or, more conveniently if a small quantity is being handled, by centrifuging through a cloth bag held in the mouth of a centrifuge tube by a rubber band. The fluid is then frozen for 10–12 hours at −10° C. On thawing, a heavy green precipitate separates, leaving a clear yellowish-green supernatant whose volume is about equal to that of the mincer extract already made. This fluid will be called " mill extract." The preliminary washing of the minced fibre is not essential, but it enhances the differences between mincer and mill extract.

Separation of Virus Preparations from the Extracts.

By centrifugal fractionation of such extracts, preparations of virus with widely different properties can be made. For this purpose we have used an air-driven centrifuge of the type described by Masket (1941), run at either 40,000 r.p.m. (R.C.F. 80,000) or 24,000 r.p.m. (R.C.F. 30,000). The ten tubes each containing 7·5 ml. of fluid are inclined at only 10° to the axis of rotation, so that the centrifugal field is relatively uniform throughout the tube. The virus particles sediment towards the side of the tube, but, from purified virus preparations, the material always slides down the wall to form a pear-shaped pellet. When fractionations are attempted the fluids should be free from fatty materials, such as leaf chromoprotein, for these stick to the walls of the tube in the form of a streak in which virus remains entangled during centrifugation ; when the centrifuge stops this partly redissolves in the supernatant.

Centrifuging for 30 minutes at 40,000 r.p.m. sediments all the virus, and concentrates it partly as a colourless, birefringent, coherent pellet, and partly as a brown uncompacted dense but mobile fluid, which we call uncompacted sediment.

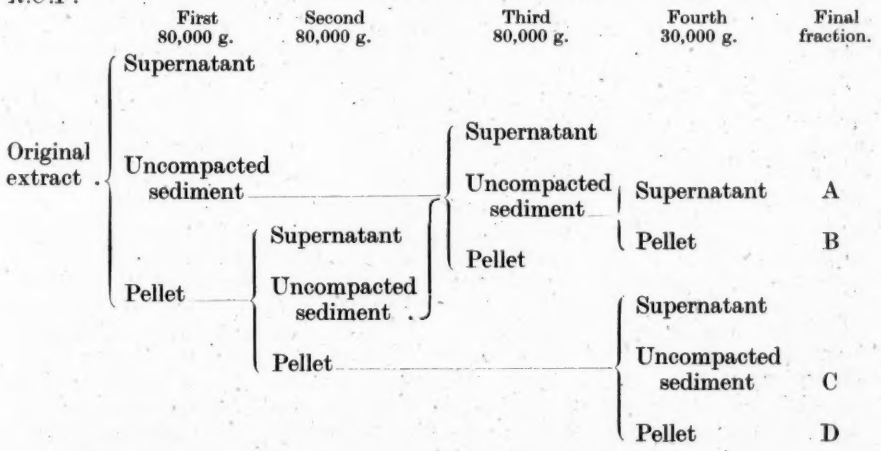
The supernatant fluids are removed carefully with a syphon so as to leave the layer of uncompacted sediment undisturbed ; the supernatants are discarded and the uncompacted layers combined. The pellets are mixed, suspended in water and centrifuged for 10 minutes at 10,000 r.p.m. after dilution to one-third of the original volume. The brownish, slightly opalescent fluid is again ultracentrifuged for 30 minutes

at 40,000 r.p.m., and the uncompacted sediments collected as before and mixed with the earlier samples. The pellets are dissolved, diluted to one-quarter of the original volume, and separated from any insoluble material by centrifugation at 10,000 r.p.m.

There are now two products, all the uncompacted sediments and the pellets that have been compacted twice. The latter contains less than 0·5 g. per litre of material unsedimentable at 40,000 r.p.m. and is ready for further fractionation, but the former needs a further sedimentation to reach this condition. It is therefore centrifuged at 40,000 r.p.m., the supernatant and the small pellet are discarded, the layer of uncompacted sediment is kept. Both products are now fractionated by centrifugation for 30 minutes at 24,000 r.p.m. The uncompacted sediment gives another tiny pellet (B), and a supernatant which merges into an uncompacted layer at the bottom of the tube (A). The pellets give three fractions which are separated, a supernatant which is little but water, an uncompacted sediment (C) and a pellet (D). If D shows any anisotropy of flow in 1 per cent solution it can be fractionated further into a pellet and supernatant by centrifuging for 30 minutes at 17,000 r.p.m. ; only the latter is used.

The course of a fractionation from starting material to end-products is illustrated in Table I ; the letters assigned to the end-products will be used for fractions whose

TABLE I.—*Scheme of the Differential Ultracentrifugations used in the Separation of Virus Preparations in Different States of Aggregation. Intensity of Centrifugation in R.C.F.*



properties are described later, so that there can be unambiguous reference to their history. All the centrifugations are done within as short a time as possible, with solutions containing less than 8 g. of solids per litre and the pH is kept between 6·5 and 7. In this type of centrifuge the surface of pellets always lies vertically, and because of their density they disperse rapidly as soon as the effective direction of gravitational acceleration changes from horizontal to vertical as the centrifuge stops. The centrifuge is therefore decelerated from 10,000 r.p.m. in 5 to 10 minutes and the tubes removed and the fractions separated as rapidly as possible.

Comparable products can be made from mincer or mill extracts, but there are consistent differences in the ratios in which the different fractions occur in the two starting materials. Table II gives figures for eight fractionations and shows that,

although there are wide variations in the ratio of compacted to uncompacted material in different batches of leaves, there is consistently more uncompacted sediment in the mincer than in the mill extract. Thus the first column of ratios covers a wide range, but the second column of ratios, which relates the mincer extract ratios to the mill extract ratios, is more uniform and all the numbers in it are greater than 1. There is a more or less continuous gradation between fractions of types A and D, and our division into four distinct fractions is arbitrary. From some leaf extracts, for example those giving the fractions whose sedimentation constants are set out in the following paper (Johnston and Ogston, 1945), the material in fraction C is larger than that in fraction B. No correction for this has been made in Table II, where the fractions are divided broadly into two groups; any such correction, however, would be small, because fractions B and C usually contain about equal amounts of material.

TABLE II.—*Yields of Pellet and Uncompacted Sediment from Mincer and Mill Extracts.*

Source.	Yield in grammes per litre in the form of—		Ratios.	
	Pellet (Fractions B + D).	Uncompacted sediment (Fractions A + C).	Pellet / Uncompacted.	Mincer ratio. / Mill ratio.
Mincer ext.	1·95	0·45	4·33	3·2
Mill ext.	3·7	2·7	1·37	
Mincer ext.	0·49	0·41	1·19	1·8
Mill ext.	0·68	1·0	0·68	
Mincer ext.	1·20	0·54	2·22	1·3
Mill ext.	1·57	0·88	1·78	
Mincer ext.	0·91	0·65	1·40	1·6
Mill ext.	1·87	2·14	0·87	
Mincer ext.	0·41	0·85	0·48	1·4
Mill ext.	0·56	1·64	0·34	
Mincer ext.	0·34	0·15	2·27	1·6
Mill ext.	0·57	0·40	1·42	
Mincer ext.	1·0	0·29	3·45	1·7
Mill ext.	1·46	0·73	2·00	
Mincer ext.	1·62	0·29	5·56	1·5
Mill ext.	3·76	1·03	3·65	

The mincer and mill extracts that form a pair were made by the method described in the text from the same batch of leaves. Batches were worked up at different dates throughout the year.

In the method of preparation described, the frozen leaves are put directly into a large volume of water so as to ensure the rapid removal of crystalloids from the flaccid leaf cells. There is a little virus in this fluid and, judging by its serological behaviour, it is in the aggregated state, but it is too dilute for isolation to be feasible. A few preparations have been made by ultracentrifuging the juice that runs out of the frozen leaves when they are pressed gently after being allowed to thaw. About one-tenth of the total virus that can be isolated from the leaf is in this fraction; it is more highly aggregated than any of the other fractions, although the aggregation can be carried still further by the methods described later. No sedimentable but uncompacted material has been found in this juice.

Methods of Testing.

Infectivity tests were made by the local lesion method, using a Latin square design on *Nicotiana glutinosa*. Precipitin tests were made by adding 1 ml. of diluted antiserum to each of a series of tubes containing 1 ml. of antigen at various dilutions. The tubes were immediately placed in a water bath at 50° C., with their fluid columns half immersed to ensure continuous mixing by convection, as previously described (Bawden and Pirie, 1937*a*, 1943). In earlier work attention was given largely to the precipitation end point obtained, for this gave a reasonably accurate measure of the quantity of virus in the antigen under test. In the present work, however, differences in end-point were obtained with preparations known to contain the same amount of virus, and different fractions behaved qualitatively very differently in precipitin tests. These differences are most simply demonstrated by comparing the behaviour of a mill extract of infected leaves before and after incubation with trypsin (Table III). Before incubation the extract showed no anisotropy of flow, and with antiserum precipitated only slowly and then over only a small range of antigen/antibody ratios. After incubation the extract showed anisotropy of flow strongly, and it then precipitated with antiserum almost immediately and over a wide range of antigen/antibody ratios. After incubation the precipitate was also of a different type, bulky and flocculent (typical " H "-type) instead of small and rather granular. When comparing different preparations, therefore, in addition to recording the precipitin titre, records were also made of the time of the first appearance of precipitation, the antigen concentration in the optimal tubes, the zone of precipitation and the character of the precipitate.

Our observations of anisotropy of flow were made simply by examining the fluids in polarized light as they are tipped from end to end of half-filled test tubes 0·5 cm. in diameter. Under these conditions the phenomenon is detectable in solutions containing as little as 0·1 g. of aggregated virus per litre. We prefer to use this rough method of comparing anisotropy of flow rather than to make measurements of the amount of birefringence built up by a given rate of shear (Lauffer and Stanley, 1938), for, in our first experiments on anisotropy of flow, we found that preparations varied greatly in the rate of shear needed to elicit a given amount of birefringence, and that if high rates of shear were used, very significant differences were missed. Furthermore, the intensity of anisotropy of flow was greatly influenced by other components in the system. It seemed, therefore, that the precision apparently lent to a set of observations by a measurement of the birefringence was likely to be spurious. This view is confirmed by the critique that Robinson (1939) has published of all measurements on anomalously flowing liquids made in capillary apparatus. In our opinion only a series of measurements made at a number of different rates of shear would give as much information about differences between two preparations as is given by a simple observation of them shaken side by side.

PROPERTIES OF CENTRIFUGALLY FRACTIONATED VIRUS PREPARATIONS.

Infectivity and Serological Activity.

Table IV shows the results of precipitation and infectivity tests made on the four fractions obtained by differential centrifugation from the mincer and mill extracts of one sample of infected leaves. These are typical of many such tests that have been made. In all, the fraction compacting at 24,000 r.p.m. (D) and that uncompacted at 40,000 r.p.m. (A) differ strikingly in their serological behaviour, and the former is highly infective, whereas the latter is almost non-infective. The fractions intermediate

TABLE III.—*Effect of Aggregation by Trypsin on the Serological Behaviour of Tobacco Mosaic Virus.*

Test antigen.	Time after mixing with antiserum.	Antigen dilution. 1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.	1 : 256.
Freshly prepared mill extract	1 hour	−	−	−	−	−	−	+	−	−
	2 hours	−	−	−	−	−	+	++	+	−
Mill extract after incubation with 0·1 % trypsin at pH 7 for 8 hours.	3 minutes	++++	++++	++++	+++	+	−	−	−	−
	30 ,,	++++	++++	++++	++++	++++	+++	++	+	−
	1 hour	++++	++++	++++	++++	++++	++++	++++	+++	++

Antiserum used at 1 : 400.

TABLE IV.—*Serological Behaviour and Infectivity of Centrifugally Isolated Fractions from Mincer and Mill Extracts.*

Fractions from mincer extract.	Time for first appearance of floccules.	Dilution of antigen in first tube to precipitate.	Zone of precipitate after 3 hours.	Infectivity. Average number of lesions per leaf at— $1 : 5 \times 10^4$.	$1 : 5 \times 10^5$.
A	3 hours	1 : 64	1 : 64	0	0
B	1 hour	1 : 32	1 : 16–1 : 128	3	0
C	15 minutes	1 : 16	1 : 1 –1 : 256	54	10
D	5 ,,	1 : 2	1 : 1 –1 : 512	116	34
Fractions from mill extract.					
A	3 hours	1 : 64	1 : 64	0	0
B	1 hour	1 : 32	1 : 8–1 : 128	1	0
C	15 minutes	1 : 16	1 : 4–1 : 256	24	5
D	5 ,,	1 : 2	1 : 1–1 : 512	85	14

Antiserum used at 1 : 400. Antigen dilution 1 : 1 = 1 mg. per ml.

TABLE V.—*Effect of Ageing at 0° C. and Heating at 60° C. on the Precipitin Reaction of Uncompacted Sediment.*

Treatment.	Time after mixing with antiserum.	Dilution of antigen (1 : 1 = 1 mg./ml.). 1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.	1 : 256.
Freshly-prepared uncompacted sediment	1 hour	−	−	−	−	−	−	+	−	−
	3 hours	−	−	−	−	−	+	++	+	−
Uncompacted sediment kept for 1 week at 0° C.	30 minutes	−	−	−	−	−	+	−	−	−
	1 hour	−	−	−	−	+	++	++	+	−
	3 hours	−	++	+++	+++	+++	+++	+++	++	+
Uncompacted sediment kept for 3 weeks at 0°C.	15 minutes	−	−	−	−	+	−	−	−	−
	30 ,,	−	−	−	+	++	++	+	−	−
	3 hours	++++	++++	++++	++++	+++	+++	+++	++	+
Uncompacted sediment heated for 10 minutes at pH 5·9 and 60° C.	3 minutes	++++	++++	+++	++	+	−	−	−	−
	15 ,,	++++	++++	++++	++++	+++	++	+	−	−
	1 hour	++++	++++	++++	++++	++++	++++	+++	++	+

in their sedimentability (B and C) are also intermediate in their infectivity and serological behaviour. Similar fractions come from both types of extract, but in general those from the mincer extracts are rather more infective.

The properties of all fractions are altered by heating at 60° C. and pH 5·9, or by incubation with trypsin. All then show anisotropy of flow even at 0·5 g. per litre, precipitate rapidly with antiserum over a wide zone, give similar titres and typical flagellar type floccules. The most striking change in serological behaviour is given by fraction A, uncompacted at 40,000 r.p.m. Heating this causes the separation of a coagulum, which contains about 60 per cent of the total solid material. The remainder has the physical and serological properties of aggregated tobacco mosaic virus, but the production of these properties is not accompanied by any increase in infectivity. The difference in infectivities between the various fractions is too great to be accounted for simply by the presence of different quantities of material other than virus. Thus about 40 per cent of uncompacted sediment may be isolated as serologically active virus, yet its infectivity may be less than one-thousandth of that of pellets compacted at 24,000 r.p.m. To cause such a reduction in infectivity, the contaminating material would need to be a powerful inhibitor of infectivity, and of this there is no evidence. First, there is no increase in infectivity when these materials are removed and, secondly, the addition of uncompacted sediment to pellet gives no great reduction in infectivity.

After such aggregating treatments as heating or incubation with trypsin, the infectivity of all the fractions is reduced by about one-half when they are compared with the original preparations at dilutions of 10^{-5} and 10^{-6} g. per litre. This is presumably because aggregation leads to a smaller number of infective units, but the change in infectivity seems small compared with the striking changes in physical and serological properties.

The virus responsible for the slight infectivity of the uncompacted sediments produces symptoms in both tobacco and *N. glutinosa* identical with those produced by virus in the other fractions. Tobacco plants have been inoculated separately with fractions A and D, and there were no significant differences between the amounts of the four fractions that could later be separated from the infected plants.

Chemical Constitution.

The main pellets compacted at 24,000 r.p.m. (Fraction D, Table I) usually have analytical compositions falling within the range we have given for tobacco mosaic virus purified by precipitation methods (Bawden and Pirie, 1937*a*), but a few have contained 0·9 per cent. to 1·2 per cent of phosphorus and 5 per cent to 7 per cent of carbohydrate—about twice the normal values. Repeated ultracentrifugation has no effect on their constitution unless they have been allowed to age *in vitro* or they have been heated or incubated with trypsin. Then highly aggregated preparations are obtained with the normal content of phosphorus and carbohydrate. This phenomenon has been noticed only with preparations made from plants grown in the winter, but we have not studied a sufficiently extensive series to define the conditions that bring it about.

The analytical composition of the intermediate fractions, B and C (Table I), also generally falls within the usual range, but that of fraction A is more variable. The figures for all these uncompacted sediments lie in the ranges nitrogen 12 per cent to 15 per cent, phosphorus 0·1 per cent to 0·7 per cent, and carbohydrate 3 per cent to 10 per cent. Part of the material in this fraction is a component of the normal leaf. but when uninfected leaves comparable with infected ones are fractionated only about

Uncompacted sediment heated for 10 minutes at pH 5·9 and 60° C. 3 minutes 15 " 1 hour 3 hours

one-fifth as much uncompacted sediment is obtained. After incubation with trypsin, the amount of material that can be obtained from this fraction with the physical and serological characters of aggregated tobacco mosaic virus varies from 10 per cent to 40 per cent in different preparations. Thus the virus in this fraction is accompanied by, or combined with, much material which has chemical properties widely different from the virus itself, but there is little evidence on the nature of the material. A preliminary study has been made of the distribution of phosphorus in a series of fractions treated in various ways to differentiate between virus-phosphorus and phosphorus in other forms. This shows that after incubation with trypsin at pH 6·8 in glucosamine buffer, all the phosphorus except that associated with serologically active virus remains unsedimentable when ultracentrifuged. Glucosamine buffer is useful when phosphorus is to be determined, because its pK is 7·8 (Miles and Pirie, 1939), and it is sufficiently stable for use in these short periods of incubation.

Physical Properties.

Aggregated tobacco mosaic virus precipitates optimally from dilute salt solutions at pH 3·4 and is soluble at pH values greater than 4, although in the absence of salts there is precipitation at pH 4·2 (Bawden and Pirie, 1937*a*). Centrifugally isolated virus behaves differently, and the different fractions differ from one another. Material that compacts at 24,000 r.p.m., fraction D, precipitates completely at pH 4·4, even in the presence of salts. The precipitates do not have the characteristic fibrous structure of aggregated virus, and they are difficult to see when separating from dilute solutions. They are highly hydrated, and even after centrifuging for 30 min. at 3000 r.p.m., the precipitate from 1 mg. of virus occupies 0·2 ml. As the pH is lowered, a denser and more easily visible precipitate separates, but it is only after standing for several hours that it develops the characteristic shimmer. Fractions such as B and C, which sediment slowly on ultracentrifugation and contain significant amounts of material that cannot be converted into aggregated virus, are precipitated at higher pH values. The precise value varies, but there is generally complete precipitation at pH 5. These fractions are not stable at this pH, and the changes that proceed are accompanied by a pH drift so that, in unbuffered solution, repeated addition of acid is needed to keep the pH at 5. After exposure to pH's between 4 and 5, and subsequent neutralization, part of a fraction that previously did not compact on ultracentrifugation at 24,000 r.p.m will do so. It is obvious from the method of separation used that these fractions differ from one another in their sedimentation constants, and measurements on 11 fractions are given in the accompanying paper (Johnston and Ogston, 1945). They also differ in the readiness with which they can be orientated. Under our conditions of testing, the fractions A, B and C do not show anisotropy of flow even in 2 per cent solution ; D shows it at this concentration, but on further dilution it disappears. The precise concentration at which anisotropy of flow is no longer readily elicited depends on a number of factors, among which are the age of the infected plants and the speed with which the fractionation, especially in its first stages, was carried through. The limit is usually between 2 and 5 g. per litre. The pellets show a similarly graded birefringence when examined in the ultracentrifuge tube without stirring : pellets like D resemble those from fully aggregated virus preparations, whereas those from B are brown and show little or no birefringence. The fact that fractions with different antecedents may have similar sedimentation rates, although their other properties are widely different, suggests that fractions differ from one another in more factors than particle size.

The process of pellet formation in the ultracentrifuge, even when not accompanied by any fractionation, leads to some aggregation even if the centrifuge is chilled and the pellet is dispersed in water immediately after centrifuging. There is still more aggregation if the dispersing is delayed for a few days.

Treatments Causing Aggregation.

All the centrifugally isolated fractions are unstable, and on ageing pass into forms that show anisotropy of flow more strongly and give precipitin reactions more characteristic of flagellar type antigens. The change occurs more rapidly in slightly acid than in alkaline conditions, and more rapidly at room temperature than at 0° C. The effects of ageing and heating on a sediment uncompacted at 40,000 r.p.m. (fraction A) are shown in Table V.

We have studied the aggregation of these fractions by exposing 0·2 per cent solutions to various conditions and observing the changes in precipitin behaviour and in appearance of anisotropy of flow. The two criteria are correlated, but in our conditions of testing cover different ranges of aggregation. Significant changes in precipitin behaviour can be detected before aggregation has proceeded far enough for the solutions to show anisotropy of flow, and by the time the solutions show significant anisotropy of flow they are fairly typical of flagellar type antigens. Further aggregation leads to little change in precipitin behaviour, but to considerable increases in anisotropy of flow. The first stages of aggregation are therefore followed serologically and the later ones by observations of anisotropy of flow. The changes are also associated with an increase in ease of sedimentation in the preparative ultracentrifuge, but measurements of the changes in sedimentation constant during aggregation have not been made.

Preliminary electron micrographs of our preparations, made by Dr. E. M. Crook and Dr. F. M. L. Sheffield, show that fractions such as A (Table I), which show no anisotropy of flow and behave like somatic type antigens, contain few rod-like particles, whereas rod-like particles predominate in fractions showing anisotropy of flow, and their average length increases as a result of treatments that increase anisotropy of flow and change the serological behaviour to the flagellar type.

Treatments leading to aggregation are conveniently studied with preparations showing no anisotropy of flow at 0·5 per cent and very little at 2 per cent (i.e. C or D in Table I). D already contains some aggregated virus, but it is most suitable for tests on aggregation as it is relatively stable. It appears to be free from those substances that cause aggregation, and spontaneous precipitates do not form in it nor do bacteria grow readily. Even so, only agents that cause aggregation in a few hours at 37° C. have been studied, for we have found no satisfactory antiseptic that does not itself cause aggregation, and products of bacterial growth are also powerful aggregating agents. Reproducible results are obtainable only with precisely controlled pH and salt content.

Aggregation is rapid in sap and is less in mincer extracts, which do not contain the juice that comes from frozen unminced leaves. The small amount of virus in this juice is highly aggregated. The reason for this is suggested by Table VI, which shows the effect of exposing the centrifugally fractionated virus to various dilutions of the supernatant prepared by centrifuging the juice from frozen unminced infected leaves at 40,000 r.p.m. to remove the virus. It will be seen that even when diluted tenfold this causes some aggregation. The juice has only about 1/20 of the protein content of the mincer extracts ; it is essentially a leaf ultra-filtrate, and ultra-filtrates made by passing infective sap through a " cellophane " membrane had similar aggre-

TABLE VI.—*Aggregating Effect of the Unsedimentable Part of the Fluid from Frozen but Unminced Leaves.*

Dilution of fluid.	Anisotropy of flow.	Time of first precipitation with antiserum.	Dilution of antigen at the optimum.
Undiluted	+++	2 minutes	1 : 1,000
1 : 1	++	2 ,,	1 : 1,000
1 : 3	+	4 ,,	1 : 4,000
1 : 10	—	8 ,,	1 : 8,000
Water	—	20 ,,	1 : 16,000

0·1 ml. lots of a 20 g./l. solution of virus fraction D were added to 0·9 ml. of fluid made up by mixing the requisite amount of juice prepared from frozen leaves as described in the text with 2 g./l. phosphate buffer at pH 5·8. This was also the pH of the juice. All contain 2 g. virus per litre and they were kept for 2½ hours at 37°. The symbols +++, ++, + correspond approximately to the anisotropy of flow shown by 1·0, 0·3 and 0·1 g./l. solutions of fully aggregated virus in M/20 pH 6 phosphate buffer.

gating effects. Fluids prepared in the same way from uninfected leaves behaved similarly.

At pH values below 5·5, anisotropy of flow increases rapidly even at room temperature, but on returning to pH 6·5–7 it is reduced or disappears, depending on whether exposure has been prolonged or short. As the pH is raised above 7, the amount of anisotropy of flow shown diminishes even with products already highly aggregated. This is reflected in the serological behaviour, for when precipitin tests are made at pH 8 precipitation is slower, and there is a greater zone of antigen excess than when tests are made below pH 7. Between pH 6 and pH 7 preparations are not apparently affected by exposure for a few hours to 37°. After partial aggregation by any of the agents that have been studied the properties shown depend on the thermal history of the product. Fully aggregated preparations show less anisotropy of flow at high temperatures than at low, but the change takes place within a few minutes, or even seconds, of the change in temperature (Bawden and Pirie, 1937*a*). By contrast, products partially aggregated by treatments at 37° C., which show moderate anisotropy of flow in 0·2 per cent solution when examined immediately, may lose this in a minute or two if cooled to 0° C. In part this is due to the smaller rates of shear attained in cold fluids, because of increased viscosity, but anisotropy of flow is not restored when the viscosity is lowered again by warming. On the contrary, anisotropy of flow which, though reduced, was still perceptible at 0° C. may disappear when the temperature is raised to 37° C. The rate at which it returns on incubation depends on the treatments used to cause the partial aggregation. The effects of cooling are also reflected in the precipitin behaviour. When tested immediately after incubation, partially aggregated preparations precipitate rapidly with antiserum over a wide range of antigen-antibody ratios, but if first cooled to 0° C. so that the anisotropy of flow has disappeared, they precipitate more slowly and give zones of antigen excess. In assessing the ability of different treatments to cause aggregation, therefore, tests were made on fluids immediately after they were removed from the incubator.

Aggregation, or partial aggregation, during incubation may be responsible for the production of double zones of precipitation which often occur when fractions such as A and B are tested serologically. Precipitation first appears with dilute antigen and is restricted to a narrow zone around the optimum, but after 2 or 3 hours in the water bath precipitation begins in the most concentrated antigen solutions, and these flocculles are typical flagellar type. Sometimes the precipitates in these tubes disappear if the tubes are left at room temperature overnight, presumably because cooling has led to disaggregation, even though the virus is combined with antibody, and has

re-established the condition of antigen excess. When again heated to 50° C. these precipitates reappear, though not immediately. No such reversible precipitation has ever been noticed with fully aggregated virus or with the small precipitates formed by dilute solutions of unaggregated preparations.

Aggregation is affected by the salt concentration in two ways; when sufficient is present it proceeds without the addition of any other material, and when insufficient is present the other agents investigated do not cause aggregation. This is illustrated, with phosphate and trypsin, in Table VII. In the absence of salts changes do go on,

TABLE VII.—*Effect of Phosphate on the Aggregation.*

Concentration of phosphate.	Concentration of trypsin.	Duration of exposure at 37°.	Anisotropy of flow.
10 g. per l.	0	1 hour	+++
5 g. per l.	0	1 ,,	++
2·6 g. per l.	0	3 hours	+
2·0 g. per l.	0·13 g. per l.	2 ,,	++
0·6 g. per l.	0·13 g. per l.	2 ,,	+
0·0 g. per l.	0·13 g. per l.	2 ,,	—

2 g./l. solutions of virus were incubated at pH 6·0 under the conditions stated. +++, etc., have the same connotation as in Table VI.

for preparations that have been incubated with trypsin alone, and which have not therefore shown any increase in anisotropy of flow, will develop anisotropy of flow within a few minutes of the addition of sodium chloride or phosphate. A close comparison of the relative efficiencies of different ions has not been made, and most experiments have been made in the presence of M/40–M/60 sodium chloride, sodium phosphate, sodium acetate or glucosamine.

Various enzyme preparations cause aggregation at pH 6·7 in M/40 phosphate or glucosamine buffer. Trypsin and chymotrypsin, purified by the method of Northrop (1939), are the most active, but the soluble and indiffusible component of commercial "trypsin" (B.D.H.), takadiastase, ribonuclease (Kunitz, 1940) and the mixed snail stomach carbohydrases also work to an extent that is roughly proportional to their protease content. Commercial pepsin also is effective at pH 7·8; when tested at pH 2·5 there is much more rapid aggregation that at the same pH without pepsin, but this aggregation is apparently reversed by subsequent neutralization. The hypothesis that the aggregation under all these conditions is an effect of proteolysis seems reasonable, but we have no definite evidence for it.

A wide range of other substances will also cause aggregation at 37° C. in the presence of salts; among these may be mentioned saturated solutions of amyl alcohol, benzene, chloroform, ether, octyl alcohol, thymol and toluene, and 0·3 per cent solutions of benzoate or salicylate and 1 per cent fluoride.

These treatments will convert part of the less rapidly sedimenting fractions, e.g. A or B in Table I, into rapidly sedimenting nucleoprotein precipitable by virus antiserum, but the fractions have a variable content of material that cannot be so converted. This extraneous material contains both protein and carbohydrate, and part of it separates as a coagulum if the fraction is heated to 70° in M/20 pH 5·7 buffer, but this treatment will not fully aggregate a preparation with a low virus content. Even at this pH, heating seems to bring about stable combination between the virus and some of the contaminants; at pH 7·0–7·5 there is pronounced combination and little or no aggregation. In a similar way, if the pH of the sap from infected leaves is adjusted

to 7·0–7·5 the amount of sedimentable material may be doubled by heating, and the pellets that separate when this heated fluid is ultracentrifuged are brown and only slightly birefringent. Presumably these phenomena are comparable with the combination that occurs when aggregated virus is heated with serum proteins (Bawden and Kleczkowski, 1941).

Full aggregation can be achieved by incubation with trypsin, but the greater the content of material other than serologically active virus the greater the amount of trypsin needed to bring about full aggregation. Fractions containing 30–40 per cent of extraneous material when incubated in 0·2 per cent solution with 0·1 per cent trypsin at pH 6·8 show the disaggregating effect of cooling most clearly, for anisotropy of flow will appear after several minutes' incubation, and rise to an apparent maximum in an hour or two. At this stage it will disappear completely on cooling to 0°, but if the incubation is continued for several hours the aggregation becomes less readily reversible, and anisotropy of flow no longer disappears on cooling.

Tobacco Mosaic Virus as an O-type Antigen.

The most obvious difference between the serological behaviour of anisotropic and isotropic preparations of tobacco mosaic virus is that shown when the two are tested over a range of dilutions against dilute antiserum. Preparations showing anisotropy of flow precipitate quickly over a wide range of dilutions and give a high precipitin titre, whereas preparations not showing anisotropy of flow precipitate only after prolonged incubation and then only over a narrow zone, giving a smaller titre and large regions of antigen excess in which no precipitation occurs. If the concentration of antiserum is increased the speed of precipitation is increased, the optimum moves to a greater antigen concentration and precipitation occurs over a wider range. The floccules produced in these conditions are dense and granular, resembling those formed by spherical antigens more than the fluffy, open floccules given by anisotropic preparations of tobacco mosaic virus.

The serological reactions of somatic and flagellar type antigens differ in other ways than in the forms of precipitate, especially in the ability of their antisera to cause precipitation after various treatments. For example, antisera to somatic antigens lose their ability to cause precipitation more easily than antisera to flagellar antigens on ageing or heating, and formolized antisera to somatic antigens do not cause precipitation, whereas formolized antisera to flagellar antigens do. From these facts it was widely believed that the two types of antigen give rise to antibodies with different stabilities. Kleczkowski's (1941*a* and *b*) work with heated antisera disproved this, and showed that heating antisera produces antibody-albumin complexes which still combine with their antigens, but whereas flagellar antigens precipitate after combination with such complexes, somatic antigens remain in solution. Experiments with tobacco mosaic virus preparations which do and do not show anisotropy of flow confirm the view that precipitation by heated or formolized sera depends on the antigen and not on the antibodies, for the former behave in every way like somatic antigens and the latter like flagellar antigens; in other words, treated antisera which will not precipitate tobacco mosaic virus in its shorter forms, precipitate it when aggregated.

After heating for 10 minutes at 80° C. at a dilution of 1 : 10 in saline, antiserum to tobacco mosaic virus failed to precipitate either uncompacted sediment or a freshly prepared mill extract, although it still precipitated these strongly after they had been aggregated and rendered anisotropic by heating at pH 5·5 or incubation with trypsin. Similarly, antiserum which had been stored at 1° C. for 10 years still precipitated aggregated virus strongly, giving a precipitin titre of over 1 : 1000, but it would only

precipitate mill extract when used at 1 : 50. With partially aggregated preparations of the virus precipitation is reduced by such treatments, but not prevented completely. This is shown in Table VIII, where the results of formolizing tobacco mosaic virus antiserum are given. Antiserum at 1 : 4 in M/20 pH 8 phosphate buffer was treated with 0·2 per cent formaldehyde for three hours at 18° C., and then diluted 1 : 10 for comparison with untreated antiserum for its ability to precipitate these preparations of tobacco mosaic virus. Formolization had little or no effect on the ability of the antiserum to precipitate a fully aggregated preparation, reduced the precipitation of the partially aggregated preparation (possibly because only the aggregated fraction was precipitated) and failed to precipitate the unaggregated preparation.

Antigens, like antibodies, can form complex aggregates with serum albumin while undergoing the preliminary stages of heat denaturation. Complexes of somatic antigens, such as serum proteins and tomato bushy stunt virus, still combine with their antibodies but are not precipitated by them, whereas complexes with aggregated tobacco mosaic virus still precipitate normally (Bawden and Kleczkowski, 1941). Preparations of tobacco mosaic virus showing no anisotropy of flow, either mill extracts or uncompacted sediments, behave like somatic antigens. It has already been shown that these change into anisotropic preparations if they are heated to 60–70° C. below pH 6 in the presence of salts. If heated at pH 7, however, there is no change in their optical properties and no coagulum separates. This heating narrows still further the zone of precipitation with dilute antiserum, and if the ratio of other proteins to virus is high, the heated solutions may even fail to precipitate with concentrated virus antiserum. Heating in the presence of salt to 80° C. at pH 7 with added albumin leads to complete loss of precipitability with virus antiserum.

The precipitin test, by determining either precipitin end-points or optimal precipitation points, has been used successfully in earlier work for quantitative measurements of tobacco mosaic virus (Beale, 1934 ; Bawden, 1943). This work was done with virus from infective sap, and which was already aggregated. From the results in this paper it is clear that the precipitin test cannot give even an approximation to the virus content of different virus preparations if their degree of aggregation differs greatly. A given weight of virus is rendered insoluble by combination with much less antibody if it is aggregated than if unaggregated, and a visible precipitate is given by much less aggregated virus. This is clearly shown by comparing the precipitin behaviour with constant antiserum of either mill extracts or uncompacted sediments before and after aggregation by heating or trypsin ; there is a factor of over sixty in the concentration of antiserum with which the solutions containing equal weights of virus precipitate optimally and a factor of about eight in the precipitation end-points. There is competition between aggregated and unaggregated virus for the available antibody, and in mixtures of the two the presence of unaggregated virus inhibits the normal precipitation of the aggregated virus. This is shown in Table IX, which gives the results of precipitin tests with mixtures containing various amounts of aggregated and unaggregated virus. From this it is clear that the apparent uniform behaviour of a virus preparation in precipitin tests, with a single optimum, is no evidence that the preparation is homogeneous.

DISCUSSION.

The conflicting results that have been published for the size and shape of tobacco mosaic virus (Pirie, 1945) were most simply explained by assuming that the virus could occur in a range of different sizes. The data presented in this paper confirm this, but show that the properties of different preparations can vary much more than was previously suspected, and they suggest that all previous work has probably been

TABLE VIII.—*Ability of Formolized Antiserum to Precipitate Tobacco Mosaic Virus in Different States of Aggregation.*

Antigen.	Time.	Control serum 1 : 50. Antigen dilution. 1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	Formolized serum 1 : 50. Antigen dilution. 1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.
Fully aggregated	2 min.	++++	++++	++++	++++	++	−	++++	++++	++++	+++	+	−
(trypsin treated)	10 „	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	++	+
Partially aggregated	10 „	−	++	+	−	−	−	−	+	−	−	−	−
(pellet at 24,000	20 „	+	+++	++	+	−	−	++	++	−	−	−	−
r.p.m.) Fraction D	90 „	++++	++++	++++	+++	++	+	++++	++++	+++	−	−	−
Uncompacted sediment	20 „	−	−	+	−	−	−	−	−	−	−	−	−
at 40,000 r.p.m.	45 „	+++	++	+	−	−	−	−	−	−	−	−	−
(Fraction A)	90 „	+++	+++	++	+	−	−	−	−	−	−	−	−

Antigen dilution 1 : 1 = 0·5 mg./ml.

TABLE IX.—*Precipitin Behaviour of Mixtures Containing Different Proportions of Aggregated and Unaggregated Tobacco Mosaic Virus.*

Antigen. Mill extract.	Heated mill extract.	Time of first precipitation.	Antigen dilution. 1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.
1	0	120 minutes	−	−	−	−	−	+	−
0·95	0·05	60 „	−	−	−	−	+	−	−
		120 „	−	−	−	+	++	++	+
0·85	0·15	30 „	−	−	−	+	−	−	−
		60 „	−	−	++	+++	++	+	−
		120 „	−	+	++	+++	+++	++	+
0·5	0·5	3 „	−	+	+	−	−	−	−
		30 „	+	+++	++++	+++	++	+	−
		120 „	++++	++++	++++	++++	+++	++	+
0	1	3 „	++++	++++	++++	+++	+	−	−
		30 „	++++	++++	++++	++++	++++	+++	++

Antiserum used at 1 : 400. The mill extract was heated undiluted for 10 minutes at 60° C. at pH 5·5.

done with virus far removed from the state in which it is produced in the infected plant. The virus seems to have few or no fixed properties, for the properties of any given product depend on its method of preparation. By different methods of purification, preparations can be made with comparable infectivities but widely different physical and serological properties; similarly, preparations can be made with widely different infectivities but similar physical and serological properties. Many of these differences in properties can be simply accounted for on the basis of a linear aggregation of a basic particle to give rod- or thread-like particles of various lengths. This would explain the change from somatic to flagellar type of precipitin behaviour and the development of anisotropy of flow; there is ample evidence that linear aggregation does occur, but there is little evidence about the precise nature of any primary particle. We have suggested before (Bawden and Pirie, 1937*a*) that this may be small and not greatly elongated. On the evidence that has accumulated since we still incline to this view, though there are undoubtedly difficulties in it. Foremost among these is the slight infectivity of the least aggregated virus fractions. So little is known about changes which destroy infectivity without altering physical and serological properties, however, that it is impossible to assess the importance of this fact.

In this work we have laid most emphasis on conditions that lead to aggregation, and from the evidence adduced it is clear that in leaf extracts aggregation is the change most likely to occur. We have shown, however, that cooling to 0° C. leads to disaggregation in contaminated, partially aggregated preparations. The slowly sedimenting material might therefore be an artifact, produced during the freezing of the leaves, for there is a greater ratio of aggregated to disaggregated material in sap from minced unfrozen leaves than in mincer extracts from the same leaves. This probably comes about because of aggregation proceeding in the sap rather than because of disaggregation on freezing, for the ratio is also great when the sap from frozen leaves is fractionated without the preliminary removal of the diffusible leaf components. Furthermore, the ratios given by mill extracts from well washed fibre are the same whether the fibre has been frozen or not. This is not conclusive, however, for there is some disaggregation when aggregated virus is mixed with fibre from healthy leaves and passed four times through the triple roller mill. This treatment, although much more severe than the single passage used in making mill extracts, has never produced material with the properties of fractions A and B; it produces some reduction in anisotropy of flow, and changes the serological behaviour slightly towards that characteristic of disaggregated preparations. We cannot exclude the possibility that the material with small particles and slight infectivity is produced from larger and more infective particles by mincing, milling or freezing, but on balance the evidence suggests that they exist in the leaf as such and are not artifacts.

The average length of the particles in a virus preparation is controlled by the past history and present environment of the preparation; it is a compromise between the forces leading to an end-to-end adhesion and the processes, such as thermal agitation, that tend to break this adhesion. Nothing is known of the forces holding the particles together in the rods. Aggregation in the more slowly sedimenting fractions is associated with the coagulation or digestion of a large amount of material other than virus nucleoprotein. In fractions such as A, which give sedimentation constants of from 20–28 S, virus nucleoprotein amounts to only 10–40 per cent of the fraction; the size of this material cannot therefore be determined, for if it were originally polydisperse, it might not show as an entity in the sedimentation diagram. On the other hand, fractions such as B contain 70–90 per cent of material that can be converted into aggregated virus, and this material, from the results given in the accompanying paper (Johnston and Ogston, 1945), must have a sedimentation constant in the range

Antiserum used at 1 : 400. The mill extract was heated undiluted for 10 minutes at 60° C. at pH 5·5.

41 to 122 S. In some leaf extracts the greater part of the material capable of reacting with virus antiserum is present in these forms. When the more rapidly sedimenting fractions such as D are incubated with trypsin, they aggregate further. We have no evidence that these changes are also associated with the removal of extraneous material from the virus nucleoprotein, but this is possible, as our tests have not been sufficiently refined to detect the loss of less than 5 per cent of the original preparations.

The various phenomena are most simply explained by postulating that the primary virus particles are unable to exist free in solution. At its poles there are probably groups which tend to combine with other materials, so that in the plant the virus may occur in forms chemically more complex than nucleoprotein. In the absence of other suitable material it seems that the particles combine with one another to form the characteristic rods, whose average length depends on the extent to which linear aggregation is impeded by extraneous material covering the polar parts of the primary particles. Some of these states appear to be more probable than others, for the ultracentrifugal data suggest that a large proportion of the virus present in some preparations has the same sedimentation constant.

This picture of extraneous material interfering with a tendency towards aggregation affords a ready explanation of the at first sight surprising fact that treatment with a proteolytic enzyme leads to the aggregation of a preparation that is predominantly protein. If these extraneous materials are destroyed, for example, by incubation with trypsin or heating, the aggregation is extensive and stable. On the other hand, if they are merely separated from the nucleoprotein, but not destroyed, they compete for combination with the active groups on the virus. Thus the reversal of incomplete aggregation by cooling could come about because of changed conditions leading to a change in the proportion of virus nucleoprotein that is combined with itself rather than with other material. This picture is, of course, largely hypothetical, but it is now well established that tobacco mosaic virus can combine with a wide range of different materials. In this paper evidence has been given that much of the virus in the leaf is associated with other leaf components, and complexes of the virus with clupein, nicotine, arginine, ribonuclease and proteolytic enzymes have been described (Bawden and Pirie, 1937*a*, 1940 ; Loring, 1942 ; Kleczkowski, 1944).

Infective virus particles presumably have a lower limit of size, but this has not been established. Of our fractionated preparations, those containing the smallest particles are weight for weight much less infective than those with larger particles. It has already been shown that this is unlikely to be accounted for by other materials present in the preparations, and it is clear that either most of the particles with the physical and serological characters of the virus are non-infective, or all the particles in these preparations have a lower capacity for causing infection. Three possible explanations can be offered for this lower infectivity : the material may be virus that has become inactivated without losing serological activity, for this is a common phenomenon *in vitro* (Bawden and Pirie, 1937*a*) ; it may be incompletely formed virus, that is it may have developed to the stage at which it has approximately the structure of a virus particle, but lacks some essential feature ; or it may be in some way malformed virus. If either of the first two explanations were correct it might be expected that the ratio between this material and the more infective fractions would vary with the length of time the plants had been infected ; if it is inactivated virus it might be expected to increase with age, if developing virus to decrease with age. We have found no such consistent variation although we have studied leaves infected for periods varying from 10 days to 10 weeks ; up to four weeks or so the total virus increases, but the ratio of the different fractions, and their behaviour, remains much the same. We have made no extensive series of comparisons of the infectivity of

similar fractions made from plants infected for different times, but those we have made have shown no striking differences. Spencer (1942) found that the duration of infection and the nutrition of the host plant affected the infectivity of centrifugally prepared virus, preparations from old lesions and from plants receiving nitrogen being from two to five times as infective as preparations from young lesions or nitrogen-deficient plants. These differences are small compared with those between our extreme fractions, and could probably be explained on the basis of his preparations containing different amounts of materials other than nucleoprotein and being in different states of aggregation. The facts that his least infective products were always those from plants containing least virus and were those giving two sedimentation constants support this interpretation.

It is not easy to decide the extent to which the preparations of virus made hitherto contain the various fractions that we have separated, but clearly they must have been heterogeneous and have contained much aggregated material. Little of the uncompacted sediment (fraction A, Table I) would occur as such, for some of this would have aggregated while the remainder would have been discarded when the ultracentrifuge supernatant was poured off. This will certainly have happened to the intermediate fractions B and C in our earlier experiments on the centrifugation of sap (Bawden and Pirie, 1937*a*), for the maximum centrifugal field was only 16,000 g., and this was attained in a rotor so much heated by air friction that convection would seriously have interfered with sedimentation. With the more intense sedimentation used in some other work, e.g. 3 hours at 60,000 g. (Ross and Stanley, 1938), 1½ hours at 50,000 g. (Loring, Lauffer and Stanley, 1938), or 1½ hours at 60,000 g. (Stanley, 1938), much of this material could have been compacted and so carried into the final preparation. In more recent preparations (Lauffer, 1944) the ultracentrifugation has been somewhat less intense. The subsequent inhomogeneity would not be expected to show in ultracentrifugal analysis, for this is not well adapted for the recognition of contaminants unless each sub-component is homogeneous and constitutes at least 5 per cent of the material present, and each of our separated fractions contains material giving different sedimentation constants. However, it is likely that the exposure to conditions in sap would have led to much aggregation before sedimentation, so that the pellet might contain few unaggregated particles though the elongated ones would be variable in their infectivity. This is even more probable with preparations made by precipitation with acid and salt, for these conditions favour aggregation, though it is likely to be still incomplete unless the sap has been heated or the preparation has been incubated with trypsin. Indeed such preparations may be the only ones that are chemically homogeneous and contain nothing but virus nucleoprotein, and aggregation may be an unavoidable sequel to purification. No detailed study of the contaminants present in virus preparations has yet been made, but it is perhaps significant that samples tested for normal leaf proteins (Chester, 1936 ; Beale and Lojkin, 1944) have been found to contain them unless they have been incubated with trypsin (Bawden and Pirie, 1937*b*).

SUMMARY.

It is shown that tobacco mosaic virus aggregates when exposed to constituents of sap and to many other agents. A method is described for extracting virus from infected leaves which greatly minimizes aggregation. The virus in such extracts is inhomogeneous, and can be separated by differential ultracentrifuging into fractions with widely different properties. The most slowly sedimenting fractions contain much material other than virus nucleoprotein ; the virus in them shows no anisotropy of flow, has serological behaviour resembling that of somatic antigens and only small

infectivity. The most rapidly sedimenting fractions contain little except virus nucleoprotein, show anisotropy of flow and have serological behaviour characteristic of flagellar type antigens.

All the fractions are unstable and readily pass into forms that sediment rapidly, show intense anisotropy of flow and have a serological behaviour characteristic of flagellar antigens. In most fractions this change is accompanied by the destruction of material other than virus nucleoprotein. It is suggested that the primary virus particle is small and not greatly elongated, and that it occurs in the plant combined with extraneous materials, the removal of which sets free groups capable of combining with one another. Reasons for the variation in infectivity of different fractions are discussed, but no definite conclusions reached.

We wish to thank Dr. A. Kleczkowski for preparing the antisera used in this work, and the Royal Society for a grant to buy the roller mill.

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THE ULTRACENTRIFUGAL EXAMINATION OF FRACTIONS OF TOBACCO MOSAIC VIRUS.

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ELEVEN samples of preparations of tobacco mosaic virus were examined in a Svedberg oil-turbine ultracentrifuge by the " diagonal-schlieren " method of Philpot (1938). In each case the concentration was 2·5 g./litre in 0·017 M NaCl, 0·009 M KH_2PO_4 and 0·005 M Na_2HPO_4, of pH about 6·5. The salt concentration was kept low in order to reduce the risk of aggregation of the protein ; this may lead to our results for the sedimentation constants being low, through salt effects, but these effects are likely to be small ; the main object of the experiments was to compare different samples. The salts were added to solutions of the virus in water immediately before use.

The results are given in the Table I, together with data on the source and nature of the samples. Samples 807 were derived from plants grown in winter, samples 841 from plants grown in summer.

TABLE I.

Sample.	Type of preparation.*	Source.	Time for first appearance of floccules during precipitation with antiserum.	Speed of rotation, rev./sec.	S_{20} (corr.) $\times 10^{-13}$.
807E	C	Mincer	15 min.	310	188, 154
807H	C	Mill	7 ,,	320	192
807B	B	Mincer	1 hour	600	45·9
807D	B	Mill	Slow	535	63·6 41·1
807J	A	Mincer	No precipitation	810	28·1 20·1
807K	A	Mill	Ditto	755	28·5
841F	D	Mincer	<5 min.	300	189
841G	D	Mill	<5 ,,	300	190, 162
841L	C	Mincer	5 ,,	300	186, (164)†
841M	C	Mill	5 ,,	300	191, 124
841D	B	Mill + mincer	2 hours	300	122

* See Bawden, F. C., and Pirie, N. W. (1945).
† This value is unreliable owing to the diffuse nature of the boundary.

The diagrams (Fig. 1) show the presence in the various samples of seven recognizable components, only two of which (S_{20} = 190 and 122 $\times 10^{-13}$) occur alone. The " components " having S_{20} less than 100 $\times 10^{-13}$ represent merely tendencies towards certain values of the sedimentation constants in otherwise polydisperse systems. The components having S_{20} of about 160 and 122 $\times 10^{-13}$ appear at first sight to be relatively homogeneous, but the degrees of spread of their boundaries compared with their sedimentation constants show that they are due to polydisperse material whose

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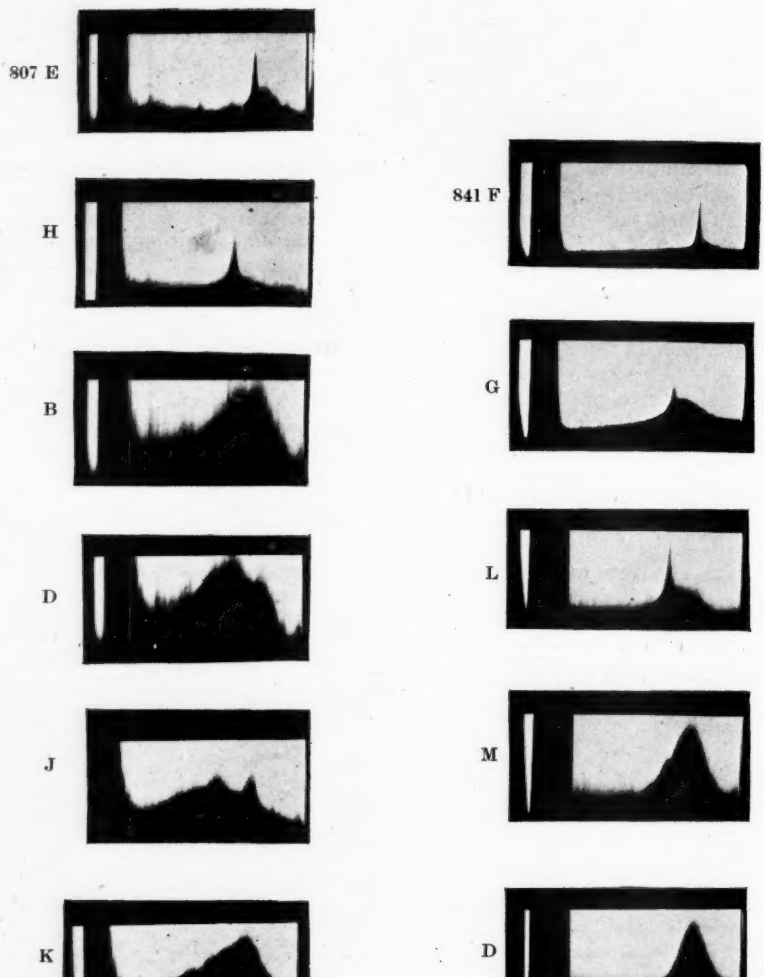

FIG. 1.—Sedimentation diagrams of the fractions described, *cf.* Table I. The diagrams are chosen to illustrate the character of the sedimenting components and are not strictly comparable with respect to times of sedimentation or optical sensitivity.

particle sizes are fairly symmetrically distributed about mean values. Nevertheless, the occurrence in different samples of boundaries having about the same sedimentation constants suggests that these mean values are not fortuitous, but represent tendencies towards certain sizes of particle.

The only component, the sharpness of whose boundary shows that it is nearly monodisperse, is that of $S_{20} = 190 \times 10^{-13}$. This is also the component of most frequent occurrence in the samples examined; it occurs accompanied by little or no heterogeneous material in 807H and 841F, and is just visible in 841M. Its boundary was actually sharper and its amount greater than appears in the diagrams, due to the boundary's moving through a considerable fraction of its width in the course of an exposure.

It was not considered to be worth while to try to obtain an accurate measure of the relative amounts of the different components by measuring the areas of the peaks in the diagrams, since the high optical sensitivity, the overlapping of the diffused boundaries and the sharpness of the fastest boundary would make the results unreliable.

Our results may be compared with those of Lauffer (1944*a* and *b*); his values of S_{20}, corrected to a concentration of 2·5 g./litre by use of his equation 2 (1944*b*), are given in Table II. It seems likely that these components are identical with the two

TABLE II.

		S_{20}(corr.) $\times 10^{-13}$.
Lauffer's preparation	A	168
,, ,,	B	190, 166

fastest observed in our samples. We agree in concluding that the component of S_{20} about 160×10^{-13} is not monodisperse; on the other hand the diagram that Lauffer gives (1944*a*) suggests that his faster component is about equally polydisperse, in contrast with ours. This discrepancy may possibly be due to his use of Lamm's scale method, which is less well suited than the diagonal schlieren method to the recording of very sharp boundaries.

These results are in general agreement with the rest of the evidence on the nature of the virus fractions. None of the more slowly sedimenting components can be identified as containing the postulated primary virus, though this is not surprising in view of the possibility that the primary virus may be combined with non-specific protein, and in view of the amount of polydisperse material present in those fractions. It is notable that the fastest fraction appears to be by much the most nearly monodisperse. The form of its boundary cannot be used as a measure of the degree of polydispersity because of the distortion of it in the record (*vide supra*), lack of knowledge of the diffusion constant and of the variation of sedimentation constant with size for highly anisometric particles, and because of the effect of the variation of sedimentation constant with concentration. It seems likely, however, that the range of particle sizes is not very great, and that, if this fraction is formed by aggregation, some factor limits the final degree or range of this aggregation fairly sharply.

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THE SELECTION OF BACTERIAL FOOD BY SOIL AMOEBAE, AND THE TOXIC EFFECTS OF BACTERIAL PIGMENTS AND OTHER PRODUCTS ON SOIL PROTOZOA.

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It has been shown that soil protozoa, especially amoebae, do not feed indiscriminately on any bacteria (Singh, 1941*a*, *b*; 1942*a*, *b*). In a preliminary survey (Singh, 1942*b*) of the edibility of miscellaneous bacteria (including plant pathogens) mostly from soil a negative correlation was observed between edibility and pigment formation. A suggestion† was made (Singh, 1942*b*) that pigment formation by bacteria exerts a protective action against protozoal attacks.

An attempt has been made in the present work outlined to find out what determines whether a bacterial strain is eaten by amoebae, and to what extent amoebae are sensitive to certain metabolic products of other organisms.

A few scattered accounts of the toxic action on protozoa of the metabolic products of bacteria, and of pigments among them, are found in the literature. Thus Birch-Hirschfeld (1934), Chatton, E., and Chatton, M. (1927*a*, *b*), Cleveland (1928), Kidder and Stuart (1939) and others have carried out some inconclusive experiments to show that bacterial pigments and metabolic products of bacteria are toxic to protozoa. Chatton and Chatton (1927*a*, *b*) claim that the chromogenic bacteria *Ps. fluorescens* and *Chr. prodigiosum* exert a cytolytic effect on a number of ciliates. Although they noted that this effect was most marked in highly pigmented strains, they drew the conclusion that pigment itself was not toxic; it was a combination of glucoside and lipoid that exerted the toxic effect upon the ciliates. They also found that saline extracts from *Ps. pyocyanea* (agar cultures) exerted cytolytic action on different ciliates. Birch-Hirschfeld (1934) obtained similar results with alcoholic extracts. They did not try to separate the various metabolic products from *Ps. pyocyanea*, and study their toxic action on protozoa.

Kidder and Stuart (1939) on the other hand, working with a ciliate (*Colpoda* sp.), claim that it is the pigment of *Chr. prodigiosum*, *Chromobacterium violaceum* and *Ps. pyocyanea* that are toxic; their conclusions are in agreement with the work carried out by the writer, but their experiments are inconclusive. They did not extract the pigments from the bacteria; they claim that when healthy ciliates and their cysts are put into heavy suspensions of *Chr. prodigiosum* and *Chr. violaceum*, the protozoa take up the colour diffusing from the bacteria.

Recently Schoental (1941) has isolated three antibacterial substances from *Ps. pyocyanea* cultures: (1) Pyocyanin, the blue pigment which appears in the young cultures during the maximal growth of bacteria. (2) A yellow pigment appearing in

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† The author is very grateful to Dr. H. G. Thornton, F.R.S., for this suggestion, from which the work on toxic effects of bacterial pigments, etc., originated.

old cultures which was found to be a derivative of pyocyanin and identified with α-hydroxyphenazine. (3) An almost colourless, chloroform-soluble bacteriolytic substance. She has compared the antibacterial activities of these substances with other disinfectants, and their action on the growth of fibroblasts in tissue culture.

1. REACTIONS BETWEEN AMOEBAE AND BACTERIA IN PURE MIXED CULTURE.

Methods.

The protozoa used in this work were two unidentified species of soil amoebae (Singh, 1941*a*). They were originally derived from single individuals, and were grown in pure culture with a species of Aerobacter as food. They are small amoebae 10–20μ long having no flagellate stage, and they are not identifiable by the characters of their cysts. In testing the availability of bacterial food the method of radiating streaks was used (Singh, 1941*a*). Sixty-three strains of bacteria* isolated from soil were used. In addition the following pigmented species, obtained from the National Collection of Type Cultures, Lister Institute, were tested : *Chromobacterium violaceum* (2537), *Micrococcus sulphurens* (1631), *Sarcina lutea* (611), *Pseudomonas pyocyanea* (1999), *Micrococcus roseus* (2683), *Torula rubra* (2627), *Chromobacterium prodigiosum* (2302), *Chromobacterium viscosum* (2416) and *Sarcina aurantiaca* (952). Other forms included two other strains of *Chr. prodigiosum* (2881, 2881C), another strain of *Ps. pyocyanea* (2650), a red bacterium (5654). The characters of bacteria 2881 and 5654 are given by Singh (1941*a*).

Throughout the experiments 1 per cent agar containing 0·5 per cent NaCl was used. This medium checks the growth of bacteria. The temperature of incubation was 20°–21° C., and bacterial cultures of the same age (2–7 days) were always used to compare their edibility by amoebae.

Results.

The bacteria tested fall into three groups ; some of them are completely eaten (readily or slowly), others are partly eaten for a few days, after which the amoebae encyst or die, while the rest are either inedible or are eaten very slightly on rare occasions. The two species of amoebae resemble one another closely in their reactions, differing only in the case of seven of the white or yellow bacteria. These are only partly eaten by the large amoebae, while they are eaten slowly but completely by the small one.

Table *I* shows the relation between pigment formation by bacteria and the feeding reactions of the two amoebae ; among a total of 56 colourless strain 71 per cent were eaten ; among 32 yellow, orange and brown 75 per cent proved suitable. One of the pink strains (*Micrococcus roseus*) was edible, while of the remainder red, violet, blue, green and fluorescent organisms were not eaten. Some among them also have an inhibitory effect on the amoebae, for when such species as *Chromobacterium violaceum* or *Chr. prodigiosum* are mixed with a strain that is readily eaten and inoculated with amoebae on non-nutrient agar plates, the amoebae in most cases die out within seven days without apparently destroying any of the bacteria.

Oehler (1916, 1924*a*, *b*) claimed that amoebae showed a preference for Gram-negative bacteria ; the results for 92 strains given in Table II show that no such correlation exists, as was pointed out from less extensive earlier data (Singh, 1941*a*).

* These bacteria were kindly given to me by Miss L. M. Crump. They comprise dominant and rare species of soil bacteria isolated from various soils. The details of their morphological and physiological characters will be published separately by Miss Crump.

TABLE I.—*Showing the Relation between Pigment Production by Bacteria and the Feeding Reaction of Two Amoebae.*

Bacteria.	Partly or completely eaten.	Not eaten.	Total.
Colourless	40	16	56
Yellow	16	7	23
Orange and brown	8	1	9
Red	—	6	6
Pink	1	2	3
Violet blue	—	2	2
Green (*Ps. pyocyanea*)	—	2	2
Fluorescent	—	1	1

(Among the 102 bacterial strains included in Table I, 29 were recorded in an earlier paper (Singh, 1942*b*); of these 17 were colourless, 7 yellow, and 5 red or violet.)

TABLE II.

	Partly or completely edible.	Inedible.	Total.
Gram-negative species	31	16	47
Gram-positive species	30	15	45

2. TOXIC EFFECTS OF BACTERIAL PIGMENTS AND OTHER METABOLIC PRODUCTS ON SOIL PROTOZOA.

The protozoa used were two amoebae previously described, a flagellate, *Cercomonas crassicauda*, and a ciliate, *Colpoda steinii*.

The species of inedible pigmented bacteria can be classified into two groups:

(1) Those that produce pigments readily soluble in water.

(2) Those whose pigments are only partly soluble or are insoluble in water.

The toxic effects of the metabolic products of the following inedible pigmented bacteria were studied: *Pseudomas pyocyanea* 2 strains, *Chromobacterium prodigiosum* 3 strains, *Chromobacterium violaceum* 1 strain, and a red bacterium (5654).

Ps. pyocyanea.

This produces water-soluble pigments. It was grown on nutrient agar for 3–7 days to allow the pigment to diffuse through the agar, and the bacteria were then scraped off. The agar was then melted and poured into a petri-dish. One or two loopfuls of edible bacteria growing on nutrient agar (2–7 days old) were spread on this agar in the form of a disc or " bacterial circle " and amoebae were inoculated in the centre of them. The amoebae died without destroying the edible bacteria, and no cyst formation could be observed. If the amoebae were inoculated into closely parallel streaks (Singh, 1941*a*) of *Ps. pyocyanea* and of an edible organism on nutrient agar, the amoebae placed in the latter streak died or encysted without destroying the edible organism in appreciable amount. Thus it is clear that a diffusible toxic substance is produced by *Ps. pyocyanea*.

Extraction of crude pyocyanin.

Ps. pyocyanea (strain 2650) was generally used because it produces more pyocyanin than strain 1999. It was grown on glycerol-peptone-agar medium in 30 to 40 boiling tube slope cultures. The medium had the following composition: Glycerol 2·5 per cent, peptone 1·0 per cent, agar 1·5 per cent. It gave a profuse formation of pyocyanin and practically no fluorescent pigment. Crude pyocyanin was extracted by chloroform from cultures about 14 days old. Chloroform was left for several hours in the cultures with an occasional shaking till nearly all the blue colour was extracted. The solution was poured, the chloroform removed by evaporation and the pigment redissolved in distilled water. This was cleared by centrifugation and finally evaporated slowly to obtain crude pyocyanin.

From this dry substance solutions of known strength were made to test the toxic effect of the crude extract on protozoa. It is not pure pyocyanin because it contains a light tea-coloured substance (to be described later), and probably α-hydroxyphenazine and other substances. The solution was blue and had a pH of about 7·6.

Toxic effect of the crude extract of pyocyanin on soil protozoa.

The toxic effects of the extract from cultures of various ages were not studied separately.

TABLE III.—*Showing the Toxic Effect of the Crude Extract of Pyocyanin on Soil Protozoa.*

Pyocyanic extract.	Big amoeba.	Small amoeba.	*Colpoda steinii.*	*Cercomonas crassicauda.*
Dilution :				
1/1000	Dead 1–2 hrs.	Dead 1–3 hrs.	Dead 1 hr.	Dead 1–3 hrs.
1/2000	,, 3–6 ,,	,, 3–8 ,,	,, 2–4 hrs.	,, 2–5 ,,
1/4000	,, 12–18 ,,	Mostly dead in 48 hrs.	,, 24 ,,	,, 24 ,,
1/8000	,, 48 ,,	Mostly alive after 48 hrs.	No effect	,, 24 ,,
1/16,000	No effect	No effect	,,	Mostly dead 48 hrs.

The effect of the crude pyocyanin extract is given in Table III. The experiments were carried out in hollow-ground slides in moist chambers, and normal NaCl was used to make the necessary dilutions. The experiments were performed in duplicate, and controls were always kept in which protozoa were suspended in normal NaCl. The medium containing protozoa also contained a little of the edible bacterium, and so it is possible that the bacteria inactivated the pyocyanin to some extent, but from the point of view of comparing the toxic effects of the various substances this is immaterial because the conditions were the same in all the experiments. The toxic effects of this substance on different numbers of protozoa were not tried; in all the experiments large numbers of protozoa were always used. To make sure that pyocyanin extract contained nothing toxic coming from the medium used to grow the cultures of *Ps. pyocyanea*, glycerol-peptone-agar was extracted with chloroform for the same period as was used in the extraction of pyocyanin. After evaporating the chloroform a little brown oily or fatty substance was left behind. This was insoluble in water and had no toxic effect on the protozoa.

The cultures were microscopically examined at various intervals of time.

From Table III it is clear that the small amoeba is more resistant to the action of the crude extract of pyocyanin than the large one ; the flagellates are more easily killed than either the ciliates or the amoebae. When the crude extract of pyocyanin is autoclaved at 15 lb. pressure for 15 minutes its toxicity is to a great extent destroyed (Table V).

TABLE IV.—*Showing the Toxic Effect of the Chemically Pure Pyocyanin on Soil Protozoa.*

Chamically pure pyocyanin.	Big amoeba.	Small amoeba.	*Colpoda steinii.*
1/1500	Dead 3–4 hrs.	Dead 3–6 hrs.	Dead 2–4 hrs.
1/2000	,, 6–10 ,,	,, 8–24 ,,	,, 6–8 ,,
1/4000	Some alive 24 hrs.	No effect 24 hrs.	,, 24 ,,
1/8000	No effect	No effect	No effect

TABLE V.—*Showing the Toxic Effect of Autoclaved Crude Pyocyanin (15 lb. pressure for 15 minutes) on Soil Protozoa.*

Autoclaved pyocyanin extract.	Big amoeba.	Small amoeba.	*Colpoda steinii.*	*Cercomonas crassicauda.*
1/750	Mostly dead 48 hrs.	Some dead 48 hrs.	Dead 6–24 hrs.	All dead 48 hrs.
1/1000	Ditto	Ditto	Dead in 48 hrs.	Mostly dead 48 hrs.
1/2000	Mostly alive 48 hrs.	No effect	Movement slowed down 48 hrs.	No effect
1/4000	No effect	,,	No effect	,,
1/8000	,,	,,	,,	,,

Toxic effect of chemically pure pyocyanin.

Pyocyanin chloride was dissolved in distilled water, and was roughly adjusted to the same pH as the crude extract (pH 7·6). The solution of pyocyanin is red when it is acid and turns blue when alkaline. Table IV shows the toxic effect of pure pyocyanin on two species of amoebae, and on *Colpoda steinii*. A comparison of Tables III and IV makes it clear that pure pyocyanin is less toxic to various protozoa than is the crude extract ; it is less toxic to the small amoeba than to the large one, as was the case with the crude extract.

Toxic effect of the light tea-coloured liquid present in the watery solution of the crude pyocyanin extract.

When a watery solution of the crude extract is passed through an L5 Chamberland bacterial candle, the blue pigment is adsorbed by the candle and a light tea-coloured liquid with a pH of about 4·6 is obtained. The blue pyocyanin passes through the candle if a solution of 5 per cent. KH_2PO_4 is added. The toxic effect of the tea-coloured liquid on protozoa was tested after adjusting its pH to roughly 7·6 (the same pH as the crude extract). It is toxic to amoebae, flagellates and ciliates. The toxicity of this liquid is increased nearly twice by autoclaving it at 15 lb. pressure for 15 minutes ; no visible change takes place in the colour after autoclaving. As the amount of

liquid obtained was so small it was not possible to find its nature, and its effect at various dilutions could not be studied. When diluted two or three times with water it still retains its toxic action. If differs in its action from pyocyanin in the following respects :

(1) Its effect on *Colpoda steinii* is somewhat different, these ciliates remaining oval after death and did not become rounded as they do in the presence of pyocyanin.

(2) Its toxicity is increased by autoclaving and not reduced.

Toxic effect of α-hydroxyphenazine on soil protozoa.

As α-hydroxyphenazine is only slightly soluble in water a saturated solution was made, and the strength of the solution was found to be nearly 1/2000. The pH was adjusted to 7·6 (the same as the crude extract), and the colour of the solution was

TABLE VI.—*Showing the Toxic Effect of α-Hydroxyphenazine on Soil Protozoa. As α-Hydroxyphenazine is Partly Soluble in Water, its Effect at Lower Dilutions could not be Tested.*

α-Hydroxy-phenazine.	Big amoeba.	Small amoeba.	*Colpoda steinii.*	*Cercomonas crassicauda.*
1/2500	Mostly alive 24 hrs.	Mostly dead 10–24 hrs.	Dead 9–12 hrs. ; burst 24 hrs.	Some dead 24 hrs.
1/3000	As above	As above	Dead 9–18 hrs.	Few dead 48 hrs.
1/4000	Some dead 48 hrs.	Dead 48 hrs.	Dead 12–24 hrs.	No effect 48 hrs.
1/8000	No effect 48 hrs.	No effect 48 hrs.	No effect 48 hrs.	Ditto

yellow. Table VI shows the toxic effect of α-hydroxyphenazine on the protozoa. By comparing Tables IV and VI it appears that the chemically pure pyocyanin is slightly more toxic than α-hydroxyphenazine. A strict comparison between the toxicity of the light tea-coloured substance and the other substances obtained from the cultures of *Ps. pyocyanea* could not be made owing to the difficulty of getting them in sufficient amount.

It is interesting to note that α-hydroxyphenazine is more toxic to the small amoeba than to the large amoeba, and the flagellates seem to be more resistant than amoebae to the action of this substance. This is contrary to what was found in the case of the crude extract and the chemically pure pyocyanin. (Compare Tables III, IV and VI.)

In addition to pyocyanin and α-hydroxyphenazine Schoental (1941) isolated a third product, an almost colourless chloroform-soluble, bacteriolytic substance. This has not yet been tested on protozoa.

Toxic effect of the fluorescent pigment on soil protozoa.

For the production of fluorescent pigment *Ps. pyocyanea* (Strain 2650) was grown in flasks containing the liquid media of Nicolle and Zia Bey (1896) and Turfitt (1936, 1937). In all these media there was a good yield of the fluorescent pigment, but occasionally traces of pyocyanin were also formed. After two weeks' growth the

medium was evaporated and the pyocyanin was removed by chloroform, in which the fluorescent pigment is insoluble. This fluorescent pigment was toxic to amoebae and flagellates. The small amoeba is more resistant to the action of this pigment than the large one, and the flagellates are more easily killed than the amoebae or the ciliates. This compares with the toxic effect observed in the case of pyocyanin.

Chr. prodigiosum (*Strains* 2881, 2881*C* and 2302).

This bacterium and the others to be described produce pigment only slightly soluble in water or insoluble.

When amoebae are inoculated in the centre of circles consisting of highly pigmented strains of *Chr. prodigiosum* on non-nutrient agar plates they are unable to move far from the place of inoculation, and within a short time begin to look unhealthy and rounded. Finally they die or encyst without apparently destroying this bacterium. To demonstrate the toxic effect of the pigment the following experiments were made : *Chr. prodigiosum* was grown on the surface of nutrient agar blocks in boiling tubes for 2 weeks at 25° C. Very little diffusion of the pigment through the agar was seen. Thin slices were cut from the top of the solid agar and melted and poured in petri dishes. Bacterial circles of an edible bacterium were made on this agar and amoebae were inoculated in the centre. The amoebae died in a few days. This effect is only seen in the agar block within $\frac{1}{2}$ in. or less from the top. When slices from $\frac{1}{2}$ in. and downwards were used, the amoebae could feed and multiply on an edible species of bacterium. Thus it seems that the slight diffusion of the pigment makes the agar toxic for protozoal growth within $\frac{1}{2}$ in. It may be that the production of exotoxin and not the pigment is responsible for the death of the amoebae, or both the exo-toxin and pigment may be responsible for the toxicity of the agar.

To rest this point *Chr. prodigiosum* (Strains 2881, 2881C and 2302) was grown on the following medium : Agar 15 g., mannitol 10 g., K_2HPO_4 0·5 g., $MgSO_4$ 0·2 g., NaCl 0·2 g., $CaCO_3$ 3 g., $CaCl_2$ 0·2 g., yeast water 100 c.c. and tap water 900 c.c. Strains 2302 and 2881 completely lost their pigment in several sub-cultures and 2881C became pale pink. These strains were spread on 1 per cent. non-nutrient agar in the form of radiating streaks (Singh, 1941*a*), and amoebae were inoculated in the centre. Strain 2881C (pale pink) is partly eaten by both the amoebae ; 2881 is also partly eaten (the small amoeba does better on this strain than the large one), and 2302 is slowly and completely eaten by the small amoeba and partly by the large one. When the non-pigmented strains were sub-cultured on nutrient agar and they remained non-pigmented, the amoebae could still eat them partly or completely as described before.

Finally the pigment was extracted with absolute alcohol from a large number of cultures on nutrient agar. The pigment, which is very insoluble in water, was dried and crushed in a glass bacterial mill with a few drops of water and several loopfuls of an edible bacterium (N16 (i)). The thick paste thus obtained was spread in the form of circles on non-nutrient agar and amoebae were inoculated in the centre. The small amoeba was able to eat the edible bacterium partly within a period of 7 days and finally encysted or died. All of the large amoebae died without destroying the bacteria in appreciable numbers.

When amoebae, flagellates and ciliates were placed in heavy suspensions of *Chr. prodigiosum* (2881) in normal NaCl, no toxic effect was observed within 48 hours. No healthy individuals or healthy cysts could be seen taking a pink colour as claimed by Kidder and Stuart (1939). *Colpoda steinii* could eat the bacteria slightly, and a few reproductive cysts were formed. The slight pink colour in the cytoplasm of the ciliates was due to the accumulation of bacteria and not to the diffusion of the pigment.

When protozoa were put in NaCl solution containing finely divided suspension of the pigment, no toxic effect was observed within a period of 48 hours. This is probably due to the fact that the pigment is very insoluble in water.

Bacterium 5654.

No diffusion of exo-toxin through nutrient agar could be demonstrated in the case of this bacterium, as the pigment is insoluble in water. When amoebae were placed in the centre of bacterial circles on non-nutrient agar, they died or encysted within a day without moving far from their place of inoculation. The behaviour of the extracted pigment with absolute alcohol was the same as in the case of *Chr. prodigiosum*.

Chromobacterium violaceum (2537).

When amoebae were inoculated in the centre of bacterial circles consisting of *Chr. violaceum* on non-nutrient agar, they moved in all directions and appeared healthy for several hours, but within a day or two they died. No exo-toxin production on nutrient agar could be demonstrated by this bacterium. The pigment extracted with absolute alcohol is very slightly soluble in water. The toxic effect of the extracted pigment was studied in the same way as in the case of *Chr. prodigiosum*. In the presence of crushed pigment the amoebae ate the edible bacteria very slightly within a period of 3–4 days and then died or encysted. The cultures were examined up to 8 days, and in no case were the edible bacteria consumed. The toxic effect was more marked in the case of the big amoeba than in the small one.

When protozoa were suspended in normal NaCl containing the crushed pigment of *Chr. violaceum* in hollow-ground slides, the protozoa were killed within a day or longer, depending on the concentration of the pigment. In very few amoebae could the pigment be observed in the cytoplasm.

When amoebae, flagellates and ciliates were placed in a thick suspension of *Chr. violaceum* in normal NaCl, no apparent effect could be seen in the small amoebae up to 24 hours, but the others were killed (the flagellates within 5–12 hours, the ciliates within 1–2 hours and the big amoeba within 4–10 hours). The ciliates could be seen actively eating this bacterium, but soon became rounded and motionless and finally burst. No staining of the cytoplasm of healthy protozoa or healthy cysts could be seen as claimed by Kidder and Stuart (1939).

3. EFFECT OF CERTAIN SUBSTANCES PRODUCED BY FUNGI (PENICILLIC ACID, CITRININ AND Ca SALT OF PENICILLIN) ON SOIL PROTOZOA.

The effect of these substances on protozoa has not been studied in detail, but it may be of interest to record a few observations. Penicillic acid and citrinin at about neutral pH have a toxic effect on amoebae, flagellates and ciliates up to a dilution of 1/1500, while the Ca salt of penicillin seems to have no toxic effect on protozoa, even in strong concentrations. A ciliate (*Colpoda steinii*) could feed on edible bacteria and reproduce in the presence of strong concentrations of the Ca salt of penicillin.

Effects produced on protozoa by toxic secretions.

In the presence of toxic substances, amoebae and flagellates became rounded and finally burst. The contractile vacuoles in the amoebae were very much enlarged, after a time depending on the concentration of the toxic substances, and cease to function.

In many amoebae no contractile vacuole can be seen in the rounded individuals; probably they burst before the cell wall bursts. The amoebae and flagellates stop their movement some time before the contractile vacuole begins to swell. The ciliates gradually become rounded and motionless in the case of most of the toxic substances tried, and no cilia can be seen in the rounded individuals; they too finally burst.

DISCUSSION.

The effect that micro-organisms and their by-products have on one another are varied and complex; it is, moreover, becoming increasingly evident that they are of great practical interest to mankind; this is true in many industrial processes, in the soil, in animal tissues, and hence in the treatment of disease. The interactions may be due to straightforward competition between organisms with the same needs occupying the same habitats, or to the fact that one group of organisms prey upon the other, or to the effect that the by-product of one may have upon the existence of the other. It is the last type of interaction, demanding close collaboration between biochemists and biologists for its understanding, which is of the greatest interest from all points of view. The reactions of free-living amoebae to various types of bacteria serve to emphasize the complicated nature of these problems, for although at first sight it may seem here to be a clear case of the relation between predator and its prey, it is well known that amoebae are not nourished indiscriminately by all bacteria, though the reasons underlying their apparent choice of food are as yet by no means clear. It has been shown (Singh, 1941*a*, *b*; 1942*a*, *b*) that certain types of pigments and exo-toxin production give a satisfactory explanation of the causes of inedibility in some of the bacterial species. The writer could not find in a large number of bacteria any particular character, such as Gram staining, motility, presence of proteolytic ferment, etc., correlated with inedibility to amoebae. It may be possible that some of the inedible species in whose association the amoebae remain alive for 7–10 days (like nodule organisms (Singh, 1942*b*)) without apparently destroying the bacteria are not digested by the amoebae. Thus protozoa may, in such cases, die or encyst owing to starvation.

During the last several years the therapeutic value of the substances produced by micro-organisms has attracted much attention of the various workers. It has been shown that there is a great possibility that some of the substances produced by micro-organisms may have a chemotherapeutic value. Unfortunately the effects of these substances have not been investigated on protozoa in detail. The study of the chemistry of certain types of pigments which have been found to be toxic to soil protozoa may lead to the discovery of substances of therapeutic importance in the cure of certain diseases caused by pathogenic protozoa.

As protozoa and other micro-organisms live in close association in intestines of human beings and animals, a detailed study of their inter-relationships, along similar lines as has been carried out by the writer, may lead to the discovery of organisms whose metabolic products may have a curative value in protozoal diseases. Moreover, the encouragement of such organisms in their natural habitat may have a protective value against the disease caused by intestinal protozoa.

Some earlier work at Rothamsted showed that the fluctuations in bacterial numbers have no correlation with moisture content, temperature or other physical conditions of soil up to a considerable limit. Thus the dominance of certain bacterial species or groups must be due to their successful competition with other micro-organisms. A detailed knowledge of the causes of dominance of bacterial species in various soils would be of considerable importance to agriculture. Amoebae may be an important

factor in bringing about qualitative changes in the bacterial flora of soil, which is more important than the quantitative changes in the bacterial population. This problem is being studied by the writer.

SUMMARY.

(1) Amoebae are extremely selective in the type of bacteria that they will eat. Bacteria fall into three groups in this respect: (*a*) those that are edible (partly or completely), (*b*) those that are inedible but whose presence in the environment is not otherwise harmful to protozoa, (*c*) those whose presence alone or in a mixture with edible bacteria is definitely toxic to protozoa.

(2) In a survey of 103 miscellaneous bacteria mostly from soil, those producing red, violet, blue, green or fluorescent pigment were inedible to the protozoa.

(3) Various methods have been devised to show that bacterial pigments of *Chr. prodigiosum*, *Chr. violaceum* and a red bacterium (5654) are toxic to protozoa.

(4) Metabolic products (pyocyanin, crude extract, α-hydroxyphenazine, tea-coloured liquid, fluorescent pigment) of *Ps. pyocyanea* are toxic to soil amoebae, flagellates and ciliates.

(5) The calcium salt of penicillin is not toxic to protozoa even in strong concentrations, while penicillic acid and citrinin seem to be toxic up to a cencentration of 1/1500.

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PATHOLOGICAL STATES DUE TO DEFICIENCY OF THE SULPHUR-CONTAINING AMINO-ACIDS.

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IN 1935 T. E. Weichselbaum reported that rats, on a diet containing only 5·2 per cent. of milk protein, suddenly became ill and, unless cystine or methionine were given immediately, died within two or three days. At autopsy changes were noted in the liver and, although their nature was not recognized, it seems evident from subsequent work that they were in fact the lesions of massive hepatic necrosis. Four years later Györgi and Goldblatt (1939), when engaged in dietetic experiments designed to produce cirrhosis of the liver, noted similar changes and recognized their nature. These observations were soon confirmed (Webster, 1941; Earle and Victor, 1942; Daft, Sebrell and Lillie, 1942); but the lesion "could not be produced at will and was not a regular occurrence" (Györgi and Goldblatt, 1939) and, in the opinion of the majority of workers, was simply a transient stage in the sequence leading to portal cirrhosis. That dietary necrosis might be distinct from dietary cirrhosis was first suggested by Daft, Sebrell and Lillie (1942), but as they were unable to produce one independently of the other, and as their description of the condition they termed cirrhosis (Lillie, Ashburn, Sebrell, Daft and Lowrie, 1942) resembled post-necrotic scarring more than true cirrhosis, their suggestion was not generally accepted. In 1944, however, Himsworth and Glynn (1944*a*) were able to show that two different hepatic lesions could be produced by means of diet. One, massive hepatic necrosis, developed suddenly after a delay of several weeks, closely resembled human "acute yellow atrophy" and led to post-necrotic scarring (Glynn and Himsworth, 1944). The other developed only over many months and was characterized by the insidious development of fibrosis throughout the liver so that ultimately a condition similar to human portal cirrhosis was produced. The former lesion was related to a low intake of protein, the latter to fatty infiltration of the liver. Diets were now available which allowed the certain production of massive hepatic necrosis uncomplicated by the lesions of portal cirrhosis. Their common feature was deficiency of sulphur-containing amino-acids, and the most effective, that in which yeast was the sole source of protein, was the most defective in such acids. Later it was found that addition of methionine (Himsworth and Glynn, 1944*b*) or cystine (Himsworth and Glynn, unpublished data) to the yeast diet prevented the development of necrosis. Unbeknown to these workers, Hock and Fink (1943) in Germany were working on similar lines. By using strains of yeast with different nitrogen-sulphur ratios they showed that the severity of the lesion increased as the dietary sulphur diminished. They also discovered the protective action of cystine, but they did not test the effect of methionine.

These results point clearly to a deficiency of cystine or methionine, or both, as the causative factor in dietetic necrosis. To determine which of these is the essential factor one or other, or both, of these substances must be eliminated from the diet. To this end synthetic diets, in which the nitrogen is supplied entirely as amino-acids, are required. Such diets were used by Womack, Kemmerer and Rose (1937). Their animals died, but no pathological findings are reported. Du Vigneaud, Dyer and Kies

(1939), using synthetic diets lacking or low in methionine, cystine, and choline, noted fatty infiltration, foci of extravasated blood, minor degenerative changes, but no definite necrosis in the liver. Their results are difficult to interpret for our purpose because of the alternation of diets, the deficiency of choline, and the presence of such heavy, fatty infiltration of the liver as has been said, in itself, to cause degeneration (Györgi and Goldblatt, 1942). The synthetic diets we used were modelled on the amino-acid composition of casein, and contained sufficient choline to prevent fatty infiltration of the liver. In the course of the investigation it became apparent that, as a result of the deprivation of sulphur-amino-acids, lesions developed in several tissues. These are described and their significance discussed.

METHODS.

Animals and their general care.—White Wistar rats from the Glaxo Laboratories' colony were used. At the beginning their weight ranged about 80 g. and their age was from 38 to 46 days. Five litters of four each were distributed among the six test groups. The animals were housed and fed separately. They were weighed individually every second day. Water was allowed freely. The temperature of the animal room was kept near to 18° C.

Amino-acid mixtures (Table I).—Five mixtures were used. The sulphur-free mixture was common to each, but four had different supplements, and by the names of these

TABLE I.—*Composition of Different Amino-acid Mixtures.*

	Intake in mg./rat/day.																			
ame of mixture.	Glycine.	dl-Alanine.	dl-Valine.	l-Leucine.	dl-Isoleucine.	l-Proline.	l-Hydroxyproline.	dl-Phenylalanine.	l-Glutamic acid.	l-Aspartic acid.	dl-Serine.	l-Tyrosine.	l-Histidine HCl (hydrate).	l-Arginine HCl.	l-Lysine HCl.	l-Tryptophan.	dl-Threonine.	Sodium bicarbonate.	dl-METHIONINE.	l-CYSTINE.
lphur-free	30	60	140	100	100	60	20	80	220	40	30	65	40	65	70	20	80	75	0	0
gh methionine	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	80	0
gh cystine	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	0	64
w methionine	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	8	0
w methionine + cystine	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	8	30

they are designated. 1·3 g. of the particular mixture, which contained several racemic acids, was given daily to each animal. This amount contains a total weight of 1 g. of the laevo-rotatory isomers. All the amino-acids were either commercial products of known purity or were prepared by one of us (A. N.). We gratefully acknowledge the assistance of the Rockefeller Foundation, who supplied us with some of the amino-acids used.* The sample of *l*-leucine was shown to be free of methionine by the methods of Sofin, Rosenblum and Schultz (1943) and of McCarthy and Sullivan (1941). It will be noted that the high-methionine and the high-cystine diets contain the same amount of sulphur, and that in the basal mixture the components are approximately in the proportion of the non-sulphur amino-acids in casein.

Casein.—In a sixth group each rat received 0·86 g. of casein daily, the amount which should yield 1 g. of amino-acids on hydrolysis.

Supplements.—All rats received the following daily supplements: Thiamine hydrochloride 20 μg., pyridoxine 20 μg., riboflavine 25 μg., calcium pantothenate

* We also gratefully acknowledge a grant from the Graham Research Fund, University of London.

100 μg., choline chloride 12 mg., cod-liver oil 2 drops, arachis oil 0·5 c.c. The unknown growth factors were supplied in the form of a yeast extract made from food yeast (torula) and kindly supplied by Glaxo Laboratories. It contained only small amounts of alkali-labile sulphur and 60 mg., corresponding to 0·5 g. of fresh yeast, were given daily. Vitamin E was not given, and in animals surviving for long periods evidence of its deficiency was found.

Carbohydrate-salt mixture.—This consisted of pure corn starch 62 parts, cane sugar 20 parts, and salt mixture 3 parts. The salt mixture was that supplied by Glaxo Laboratories for metabolic experiments.

Feeding.—The aim was to keep the intake of amino-acid mixture, or casein, constant. Each day each animal received the prescribed amount of these, and the total food intake was adjusted, either to the animal's appetite, or in paired feeding experiments, to the control group, by appropriate variations in the amount of carbohydrate-salt mixture supplied. The maximum amount of food allowed daily was 12·0 g., made up of 1·3 g. of amino-acid mixture (or 0·86 of casein), 10·2 g. of carbohydrate-salt mixture (or in the case of the casein diet, 10·6 g.), 0·5 c.c. of arachis oil, and the various small supplements. Rats taking less than 12·0 g. daily were fed to capacity, unless they were members of the control group in a paired feeding experiment, when they were fed to the capacity of the test group.

*Haematological examinations.**—Haemoglobin was estimated by the Haldane method against a solution standardized by the National Physical Laboratory at 14·8 g. of haemoglobin/100 c.c. Estimations were made in duplicate. The mean cell diameter was estimated by two independent observers using the halometer method. Reticulocytes were determined by the wet method, using Cresyl Blue as the stain. Leishman's stain was used for blood and marrow smears.

Plasma protein estimations.—These were made by the densitometric method of Phillips, Van Slyke, Dole, Emerson, Hamilton and Archibald (1945), using the factor 360.

RESULTS.

Growth.

Fig. 1 shows the growth curves of rats on the high-methionine diet, and of those on the casein diet, when both groups were receiving equal and constant amounts of food to the value of 50 calories daily. It can be seen that growth on the synthetic diet is excellent, and actually better than on the casein diet. The close correspondence of the growth curves for individual animals in the same group is noteworthy, and justifies the use of curves showing the average change of weight in the other figures. Fig. 2 shows the average growth curves for the group of rats on the sulphur-free diet, and for the group on the high cystine diet in the period before pathological lesions appeared. Each animal received approximately 25 calories a day. Both groups lost weight, but the high cystine group more slowly. Fig. 3 gives the average growth curves for the low-methionine and low-methionine + cystine groups, the members of which each received 38 calories daily. The low-methionine + cystine group gained more weight than the low-methionine group.

Appetite.

In the absence of methionine the animal's appetite is greatly impaired, and this impairment cannot be cured by cystine. Rats receiving the sulphur-free diet ate only about 5½ g. of food daily ; those on the same mixture plus 64 mg. of cystine daily

* We are indebted to Miss D. E. Harding for assistance in these examinations.

(high cystine diet) ate the same amount; but those on the same mixture plus only 8 mg. of methionine (low-methionine diet) ate over 3 g. more food each day (Fig. 4). The effect of methionine deprivation on appetite appears with surprising speed. On

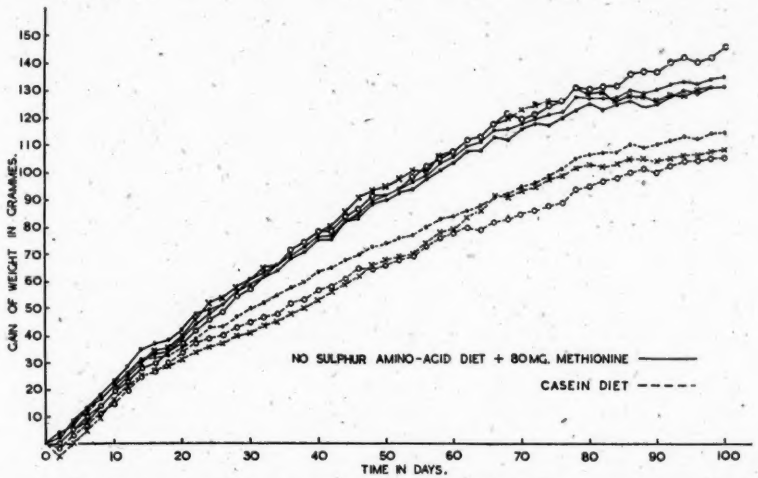

FIG. 1.—Showing the rate of growth of litter mates on isocaloric diets containing the same amount of nitrogen, but in one of which the dietary nitrogen is supplied as casein, in the other as an amino-acid mixture containing 80 mg. methionine/rat/day. The corresponding litter mates are indicated by the same point symbol, thus o—o and o- -o relate to litter mates on the amino-acid and casein diets respectively. Comparison with Figs. 2 and 3 shows that the inclusion of 80 mg. methionine/rat/day in the amino-acid mixture promotes normal growth.

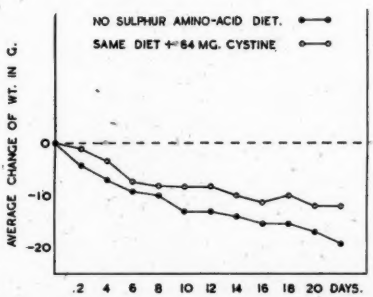

FIG. 2.—Showing the average growth curves for the three rats on the diet with no sulphur-containing amino-acids and the three rats on the same diet supplemented with 64 mg. of cystine/rat/day. The food intake was similar in the two groups (see Fig. 4). Cystine alone does not promote growth. The record is terminated at 20 days because after this time lesions developed in the rats on the sulphur-free diet.

changing rats from a normal diet to the sulphur-free diet the food consumption on the first day averaged 10·4 g. and on the second 4·7 g.; to the high-cystine diet, 11·4 g. on the first day, 5·2 g. on the second.

Survival.

All the rats on the casein, high-methionine, and low-methionine plus cystine groups survived in good health for more than 100 days, when they were either killed or used for other purposes. Of the three rats on the high-cystine diet two were killed, when

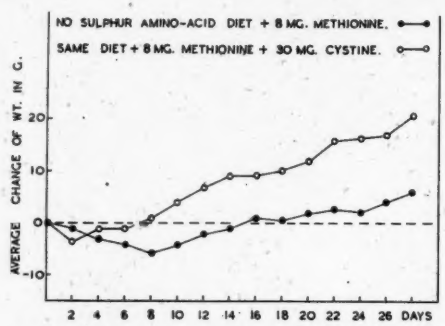
NO SULPHUR AMINO-ACID DIET + 8 MG. METHIONINE.
SAME DIET + 8 MG. METHIONINE + 30 MG. CYSTINE.
AVERAGE CHANGE OF WT. IN G.
20
10
0
-10
2 4 6 8 10 12 14 16 18 20 22 24 26 DAYS

FIG. 3.—Showing the average growth curves for four rats on the no sulphur amino-acid diet plus 8 mg. methionine/rat/day, and three rats on a similar diet but containing, in addition to 8 mg. methionine/rat/day, 30 mg./cystine/rat/day. In the presence of a small amount of methionine addition of cystine accelerates growth. The record is terminated at 27 days because after this time lesions developed in the rats on the diet without cystine.

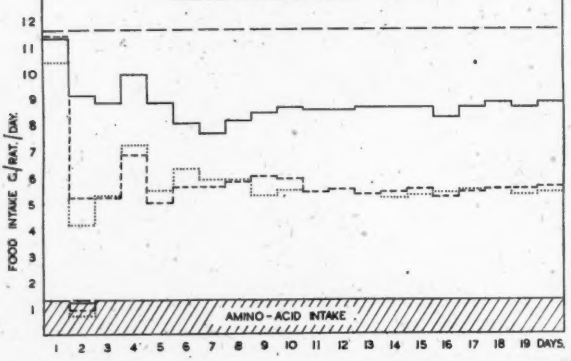

FIG. 4.—Showing that in the absence of sulphur-amino-acids appetite is greatly impaired; that this impairment is not remedied by addition of cystine; but that methionine, in supplements as small as 8 mg./rat/day, increased food consumption. Large supplements of methionine, such as 80 mg./rat/day, resulted in complete consumption of the food offered. The speed with which lack of methionine impairs appetite is noteworthy.

dying, on the 58th and 99th days respectively, and the third was found dead on the 147th day. Of the three on the sulphur-free diet two were killed when ill on the 29th and 34th days; the third was found dead on the 58th day. And of the four animals on the low-methionine diet three were killed, when evidently dying, on the 35th, 39th and 45th days, and the fourth was killed on the 104th day.

Necrosis of the liver.

Massive hepatic necrosis developed in two out of three animals on the sulphur-free diet in 34 and 58 days respectively, and in three out of four animals on the low-methionine diet (8 mg./rat/day) on the 35th, 39th and 45th day respectively. The remaining animal on the sulphur-free diet looked so ill on the 29th day that it was killed, but no necrosis was found ; the remaining one on the low methionine diet was killed on the 104th day, and showed post-necrotic scarring. One animal on the casein diet, killed on the 100th day, showed a scar of healed necrosis in the liver. Two rats which had been taking the high-methionine diet for 118 days were given the sulphur-free diet. Massive hepatic necrosis developed in 8 and 22 days respectively. One animal which had received the casein diet for 130 days and was then given the sulphur-free diet died 18 days later from massive hepatic necrosis. No hepatic lesions of any kind developed in the animals on the high-cystine, the high-methionine, and the low-methionine plus cystine mixtures. Thus massive hepatic necrosis develops when sulphur amino-acids are lacking, and is prevented when either cystine or methionine is given in adequate amounts. Its development is not related to loss of weight, for the animals on the high cystine diet lost as much weight as those on the sulphur-free diet, but showed no necrosis ; while those changed from the high-methionine diet to the sulphur-free diet died of the lesion before their weight had fallen to that at the beginning of the experiment.

Detailed chemical analyses of the livers were performed. The results will be reported in detail elsewhere. Here it need only be said that the necrotic and the normal livers showed similar chemical changes to those previously reported (Himsworth and Glynn, 1945), and that none contained abnormal amounts of fat.

Serum proteins.

The total serum protein level fell in those animals whose diet contained no methionine and remained normal in all others. The value in the former was 3·63 g./100 c.c. of serum with a standard deviation of the series of $\pm$ 0·30 ; in the latter 5·50 g./100 c.c. $\pm$ 0·37. There was no relation between the serum protein level and liver damage. Thus all three animals on the high-cystine diet showed hypoproteinaemia and none had hepatic necrosis ; all four animals on the low-methionine diet showed normal values and all had hepatic lesions. Hypoproteinaemia was correlated only with methionine deficiency ; and amounts of this substance as small as 8 mg./rat/day were sufficient to prevent it.

Anaemia.

Rats receiving any of the diets containing methionine showed no changes in the blood or bone marrow ; rats receiving diets devoid of methionine showed in the peripheral blood a marked fall in haemoglobin and erythrocytes and a pronounced increase in reticulocytes, and in the bone marrow an increase of the erythroblastic cells (Table II). The anaemia tended to be macrocytic and hyperchromic, and in stained films poikilocytosis, anisocytosis, and polychromasia were noted. The reaction in the bone marrow showed increased activity at the Type A normoblast stage (Israels' classification, 1939) ; classical megaloblasts of the pernicious anaemia type were not seen. Considerable deposits of iron were found in the spleens of animals on all the diets, but were less marked in the groups receiving diets without methionine. This we attribute to all receiving a generous supplement of iron, and to the consumption of iron being less in those animals whose appetite was impaired by lack of methionine.

TABLE II.—*Changes in the Formed Elements of the Blood and Marrow.*

	Methionine Diets.			Methionine-free Diets.						
					Individual figures.					
					Sulphur-free.			High cystine.		
	Number of animals.	Mean.	S.D.*	Mean.	(a.)	(b.)	(c.)	(d.)	(e.)	(f.)
Blood :										
Haemoglobin % : Beginning	14	96	± 9·3	101	80	105	106	124	93	97
End	14	101	± 13·4	50	72	10	85	80	13	40
Erythrocytes : Millions— End	13	10·16	± 1·39	4·90	6·0	—	5·6	8·4	0·7	3·8
Colour Index† : End	13	0·99	± 0·09	1·31	1·20	—	1·51	0·95	1·86	1·02
Mean cell diameter : End	13	5·85	± 0·25	6·16	6·1	—	6·2	5·8	6·2	6·5
Reticulocytes % : End	13	2·8	± 2·7	25·0	13·0	—	12·5	9·7	40·0	50·0
Leucocytes : End	12	9150	± 4200	5425	8000	—	9000	5000	4400	700
Marrow :										
$\frac{\text{Nucleated erythroblastic cells}}{\text{Total nucleated cells}} \times 100$	8	23·0	± 6·2	49·3	43·0	70·0	—	—	48·5	36·5

* S.D. = Standard deviation of the series.

† The colour index was calculated on the basis that the normal erythrocyte count in our rats was 10 million and the normal haemoglobin value 100 per cent (Haldane). The counts on rats "c" and "d" were obtained after death. The former died from acute hepatic necrosis, a condition known to give rise to haemoconcentration ; the latter with severe infection of the middle ears on the 147th day. The considerable variation in the values for rats on methionine-free diets must, at least in part, be attributed to such animals dying from causes other than anaemia while in the course of developing the blood changes.

Bartonella infection was sought for, but not found.

In the anaemic animals the number of leucocytes in the blood appeared to be decreased, but the number of observations is too small to establish this with certainty.

Anaemia developed more rapidly in animals on the sulphur-free diet than in those on the same diet supplemented with 64 mg. of cystine daily. Considerable variation occurred in the rate of its development in different animals on the same diet. Thus, in rats on the high-cystine diet, the haemoglobin fell 46 per cent. in 139 days in the first rat, and 80 per cent. and 57 per cent. in 91 days in the second and third rats respectively. But before the haemoglobin falls changes in the erythrocytes can be detected. Three rats which had received the low-methionine plus cystine diet for 141 days were changed to a diet which differed only in that the 8 mg. of methionine had been removed. Eight days later, although the haemoglobin level was still steady at over 100 per cent. the erythrocyte counts had fallen 2·1, 2·3 and 1·8 millions respectively, and the colour indices had risen 0·22, 0·18 and 0·04. In four rats on adequate intakes of methionine and whose haemoglobin ranged between 92 per cent. and 80 per cent., the average reticulocyte count was 2·7 per cent. In three rats on methionine-free diets, whose haemoglobin level fell within the same range, the average reticulocyte count was 11·7 per cent. These changes occur when methionine is lacking; they are uninfluenced by cystine, but prevented entirely by doses of methionine as small as 8 mg./rat/day.

Urine pigments.

Rats on the diet devoid of sulphur-amino acids began, during the second week, to pass a urine that turned dark brown on standing. Incorporation of cystine or methionine in the diet prevented the excretion of the pigment. The nature of the pigment has not yet been finally established, but it has been found that such pigmented urines give positive reactions for homogentisic acid. In view of the development of anaemia in certain groups of rats the urines were examined for porphyrins. Professor C. Rimington kindly carried out the estimation and found no evidence that these were significantly increased.

Bile pigments were detected in the urine of some, but not all, rats with hepatic necrosis. They were absent in those rats in which a fulminant lesion caused death within a few hours. In others intermittent biliuria was noted on several occasions before death, and in these evidence was found at autopsy of preceding attacks of hepatic necrosis.

DISCUSSION.

Recent work has greatly extended our knowledge of the metabolism of the sulphur-amino-acids. Until 1932 cystine was considered the only sulphur-containing essential amino-acid, and methionine, which had been discovered by J. H. Mueller in 1923, was not considered to be an important dietary constituent. Almost simultaneously, however, two groups of workers (Weichselbaum, Weichselbaum and Stewart, 1932; Jackson and Block, 1932) found that methionine can replace cystine as a growth stimulant in a diet in which the limiting factor is the content of sulphur amino-acids. Later it was shown (Braund, Cahill and Harris, 1935) that methionine and cysteine, given to a cystinuric patient, increased the excretion of cystine, while cystine and glutathione were completely oxidized. The conversion of methionine to cystine in rats was demonstrated by Womack, Kemmerer and Rose (1937) using a synthetic diet, and by White and Beach (1937–38) using a methionine-poor protein, arachin. Both groups of workers showed that methionine alone will support growth, while cystine

will not, thus demonstrating that methionine is an essential amino-acid, and that its conversion to cystine is apparently irreversible. Direct demonstration of this conversion was given by the demonstration of cystine deposition in the tissues of animals on diets containing methionine but little cystine, and this was studied quantitatively by Rose and Wood (1941) using a synthetic diet containing no cystine. Studies with radioactive and labelled elements have recently shown that the S atom of methionine is transferred to cystine (Tarver and Schmidt, 1939, 1942), but that the carbon chain is not incorporated in the cystine molecule (Du Vigneaud, Kilner, Rachele and Cohn, 1945). Thus any diet which contains methionine inevitably ensures a supply of cystine to the tissues. To some extent cystine enables the body to use methionine more economically. By itself cystine cannot support growth, but if methionine is given in suboptimal amounts, addition of cystine stimulates growth. When, however, the methionine content of the diet is 0·6 per cent., or even greater, addition of cystine has no such effect (Womack and Rose, 1941). No indication of the conversion of cystine to methionine has ever been produced ; all the evidence is against such a conversion being possible.

The satisfactory growth and the absence of pathological findings in the rats reared on the high-methionine diet show clearly that a sulphur-free diet supplemented with 80 mg. of methionine daily is entirely adequate. The results provide further confirmation, if any were needed, that cystine is not essential if sufficient methionine is given. The enhanced growth rate and absence of lesions seen when the low-methionine diet is supplemented with 30 mg. of cystine daily illustrates the methionine-sparing action of cystine.

The most unexpected findings in the present investigation were the anaemia and the hypoproteinaemia. These do not develop in the partial sulphur amino-acid deficiencies resulting from diets containing proteins relatively deficient in these acids (Himsworth and Glynn, 1944*a*) ; they only appear when deficiency is extreme. Both lesions are present together. In the present experiments in which they were produced three variables must be considered : diminished carbohydrate calories, diminished intake of iron, lack of methionine. Anaemia did not develop on diets supplying the same number of carbohydrate calories, but in which the nitrogen was supplied by a protein deficient in, but not lacking, methionine (Himsworth and Glynn, 1944*a*). In those experiments the intake of iron was less than in the present and no iron deposits were found in the spleen, although anaemia was absent (Himsworth and Glynn, 1939–42). Such deposits were present in all groups of our present animals, indicating that the intake of iron was more than adequate. The anaemia has not the morphological characteristics of that seen in iron deficiency. The hypoproteinaemia and the anaemia only developed in the absence of methionine ; they were unaffected by cystine, but completely prevented by small daily supplements of methionine. The conclusion is that the hypoproteinaemia and the anaemia observed in these experiments are the result of methionine deficiency. This should not, however, be taken to mean that hypoproteinaemia and reduction in haemoglobin or red blood cells are regarded as specific evidence of deficiency of methionine and of no other amino-acids. These manifestations have also been observed in lysine (Harris, Neuberger and Sanger, 1943), and in phenyl-alanine deficiency (Maun, Cahill and Davies, 1945) ; but in neither case was the character of the anaemia determined.

This conclusion is somewhat at variance with Robscheit-Robbins, Miller and Whipple's views on hypoproteinaemia (1943). Using protein depleted dogs they found that cystine appeared more effective than methionine in curing this condition. Our results are concerned only with prevention of hypoproteinaemia, but they lead to the opposite conclusion. The type of anaemia resembles the human nutritional macro-

cytic anaemia, not only in its morphology* and in its association with hypoproteinaemia, but also in its relation to a deficient intake of dietary protein (Wills and Mehta, 1929–30; Wills, 1933–34; Wills and Evans, 1938; Trowell, 1942, 1943; Upadhyay, 1944; Taylor and Chhuttani, 1945).

Typical, massive hepatic necrosis develops in the absence of methionine and cystine, and is prevented by the incorporation of either into the diet in adequate amounts. The experiments with rats receiving a small amount of methionine without cystine show that 8 mg. of methionine daily is insufficient to protect against the lesion. The normal fat content of the liver, even in the animals on the methionine-free diets, shows that the supplement of 12 mg. of choline chloride daily provided sufficient lipotropic factors, and excludes any possibility that fatty infiltration may be a causative agent in the production of the hepatic lesions. These results demonstrate clearly that experimental massive necrosis of the liver is a deficiency disease due to lack of sulphur-containing amino-acids. The question remains, however, which of the two amino-acids, cystine or methionine, is primarily responsible? A direct proof is impossible because of the known facts concerning the metabolism of these acids; the conversion of methionine to cystine so that inclusion of methionine in the diet is necessarily equivalent to giving a mixture of methionine and cystine; and the apparent effect of cystine in economizing the use of methionine.

No necrosis developed in the rats receiving 64 mg. of cystine, but no methionine daily. This can be interpreted to mean either that cystine is the essential preventive or, alternatively, that methionine is the preventive agent and cystine enables the body to eke out its stores of methionine. But it has also been shown that, under the conditions of our experiments, methionine deficiency produces anaemia and hypoproteinaemia. The rats on the diet with cystine but no methionine showed these lesions, but no necrosis; the rats on the diet containing 8 mg. of methionine, but no cystine, showed necrosis but neither hypoproteinaemia nor anaemia. The deduction is that hepatic necrosis is due to cystine deficiency. This deduction is supported by the following observations: One of the animals on the casein diet showed, when killed, post-necrotic scarring of the liver. It had received 860 mg. of casein daily, which amount contains approximately 30 mg. of methionine and 2.5 of cystine. The animals on the low-methionine plus cystine diet received 8 mg. of methionine and 30 mg. of cystine daily; none developed necrosis. Bromobenzene, given to rats on a 6 per cent. casein diet, inhibits growth, and in some animals produces necrotic lesions in the liver. When cystine or methionine is given to such animals growth is resumed. It is thought that bromobenzene, by combining with cystine to produce mercapturic acid, leads to cystine deficiency (White and Jackson, 1935). Rats given large doses of nicotinamide cease to grow and develop fatty but not necrotic livers. The fatty livers are prevented by choline, but methionine is required for resumption of growth. These results are interpreted to mean that methionine is intimately involved in the methylation of nicotinamide, and that the cessation of growth is due to a conditioned methionine deficiency (Handler and Dann, 1942). Weichselbaum (1935) noted that although both methionine and cystine were curative to rats when they first fell ill with hepatic necrosis, cystine alone would save moribund animals. This suggests that moribund animals are either unable, or do not survive long enough, to produce

* We have described the bone marrow in our rats as showing an early normoblastic hyperplasia. Human nutritional macrocytic anaemia is usually said to show a megaloblastic hyperplasia of the bone marrow; but it is evident, as the following quotations show, that the megaloblasts in this condition are not only few, but differ from the classical megaloblasts seen in pernicious anaemia: "In nutritional macrocytic anaemia normoblastic erythropoiesis vastly predominates"; "the N.M.A. megaloblasts approximate more to normoblasts"; and, "Many will consider that they are normoblasts" (Trowell, 1942). The cells in our animals had the size of megaloblasts, but the structure of normoblasts.

cystine from methionine. All this evidence points to cystine deficiency as the cause of dietetic massive necrosis of the liver. We know of only one piece of evidence which might have another significance. Miller, Ross and Whipple (1940) state that, in protein depleted dogs, methionine has greater protective action than cystine against necrosis of the liver due to chloroform. It should be remembered, however, that protein-depleted dogs are both anaemic and hypoproteinaemic—conditions which our results suggest would be benefited by methionine.

Lastly, there remains the observation that rats on a diet devoid of sulphur amino-acids come to excrete a chromogen identical with, or closely similar to, homogentisic acid. All that can be said at present is that this is due either to lack of cystine or to an extreme degree of methionine deficiency, or to both, and to point out that cystinuria and alkaptonuria may occur together clinically (Garrod, 1923).

SUMMARY.

1. Using diets in which all the nitrogen is supplied in the form of amino-acids, the effects on rats of deficiency of either cystine or methionine, or both, have been investigated.

2. Rats on a diet, devoid of both cystine and methionine, waste, lose appetite, develop anaemia, hypoproteinaemia and massive hepatic necrosis, and excrete in the urine a chromogen similar to, or identical with, homogentisic acid.

3. Given the same diet supplemented with cystine the animals waste, lose appetite, and develop anaemia and hypoproteinaemia ; but they do not develop hepatic necrosis or excrete the chromogen.

4. On a diet supplemented with small amounts of methionine, but without cystine, the rats show moderate growth and appetite but die with massive hepatic necrosis. They do not develop anaemia or hypoproteinaemia, neither do they excrete the chromogen.

5. On a diet supplemented with large amounts of methionine, but containing no cystine, the animals grow excellently and show no pathological lesions whatever.

6. The anaemia, which develops in animals receiving no methionine, is severe, shows anisocytosis, polychromasia and poikilocytosis, tends to be hyperchromic and macrocytic, is accompanied by a conspicuous reticulocytosis, and is associated with proliferation of type A normoblastic cells in the bone marrow. It develops despite an adequate intake of iron salts.

7. The results are interpreted in the light of modern knowledge on the metabolism of the sulphur amino-acids.

8. It is concluded that a deficiency state corresponding to lack of each of the sulphur-containing amino-acids can be distinguished.

(*a*) Methionine deficiency, in its milder degrees, produces anorexia, arrest of growth and even wasting ; in its more severe degrees hypoproteinaemia and a special type of anaemia.

(*b*) Cystine deficiency causes massive necrosis of the liver.

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BIOLOGICAL INVESTIGATIONS ON PROACTINOMYCIN.

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From the Sir William Dunn School of Pathology, Oxford.

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AN antibiotic, proactinomycin, a product of *Proactinomyces* (*Nocardia*) *gardneri*, was described by Gardner and Chain in 1942. The substance was active at a high dilution (1 : 500,000 or more) against the Gram-positive cocci, *B. anthracis* and *N. meningitidis*, and considerably less active against the Gram-negative bacilli, and it was not very toxic to animal tissues. This paper, which records a more detailed investigation of its biological properties, is based on work with material of about the same purity as before, different batches containing probably from 70 to 90 per cent. of proactinomycin. The material was prepared by the method of Gardner and Chain (1942). Chemical investigations by F. J. Philpot will be published elsewhere.

EXPERIMENTAL.

1. *Pharmacology.*

Action on cardio-vascular system.

Action on the isolated perfused cat heart.—There was a marked slowing of the rate unaffected by the addition of atropine, a slight diminution in the force of the heart, and dilatation of the coronary vessels followed by some coronary constriction. A single injection of 1·6 mg. of proactinomycin produced the effects only in a slight degree ; after 8 mg. they were much more marked, but recovery was complete (Fig. 1).

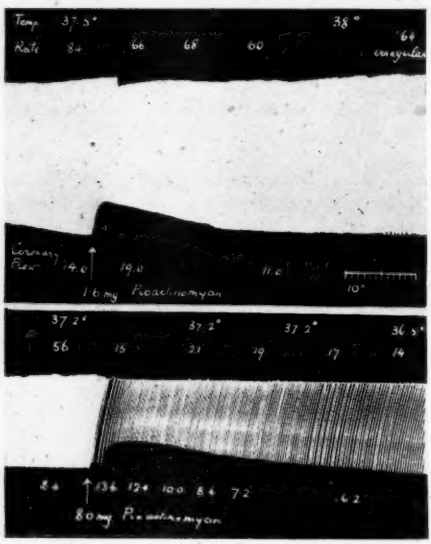

FIG. 1.—Isolated cat heart perfused with proactinomycin. Record of rate and amplitude of beat and figures for coronary flow.

(We are much indebted to Prof. J. H. Burn for performing this experiment.)

Effect on the blood pressure of the cat.—In three cats under chloralose the blood pressure rose about 30 mm. Hg following doses of 16 to 20 mg. of proactinomycin per kg. The pressure rose steeply in the first three or four minutes after injection, was sustained for about half an hour and then fell slowly, reaching its initial level in about 1 hour (Fig. 2). This prolonged rise of blood pressure, in view of the effects on the

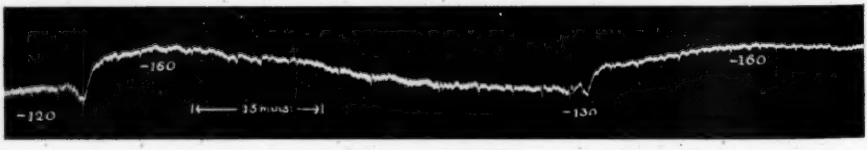

FIG. 2.—Blood-pressure record of cat under chloralose. Two intravenous injections of 50 mg. proactinomycin (20 mg. per kg.). Both injections followed by prolonged rise of blood pressure.

perfused heart, would most probably be due to vasoconstriction caused by central or peripheral stimulation, or both. The same rise was obtained under nembutal anaesthesia (1 cat), after 12·5 mg. per kg., though on repeating the injection (15 mg. per kg.) a fall of blood pressure was seen in this animal (Fig. 3). In a decerebrate cat

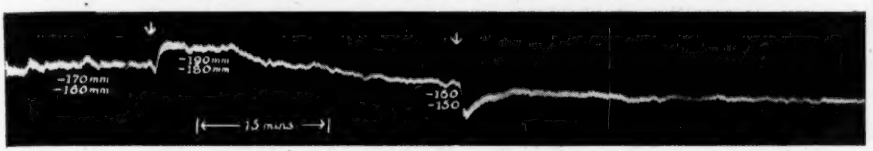

FIG. 3.—Blood-pressure record of cat under nembutal. Intravenous injections of 50 and 60 mg. proactinomycin (12·5 and 15 mg. per kg.) at the first and second arrows respectively. First injection followed by a rise, second by a fall.

(which received 17·5 mg. per kg.) there was a slow rise of pressure after a brief fall; the pressure had returned to the initial level again in about half an hour (Fig. 4).

One of the chloralosed cats in which a rise of blood pressure had been noted after 16 mg. per kg. intravenously was decapitated, one and a quarter hours later. The

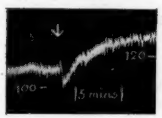

FIG. 4.—Blood-pressure record of decerebrate cat. 35 mg. proactinomycin (17·5 mg. per kg.). Shows rise of blood pressure.

same dose was given again after another three-quarters of an hour. There was a fall of blood pressure followed by slow recovery—an effect which was repeated by a further injection of proactinomycin, but not by one of saline. Two preparations decapitated under ether anaesthesia which each received two injections (14 to 24 mg. per kg.) similarly showed a sharp fall with recovery up to, but not above, the original level

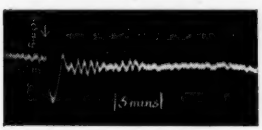

FIG. 5.—Blood-pressure record of decapitate cat. 50 mg. proactinomycin (21 mg. per kg.). Immediate fall of pressure with subsequent restoration; no rise as when vaso-motor centre intact.

(Fig. 5). It would thus appear probable that proactinomycin exerts a stimulating effect on the vaso-motor centre, causing a fairly prolonged rise of blood pressure.

Effect on the heart rate.—The slowing of the heart noted in the isolated preparation was found also in these animals. Thus in one cat under chloralose (which received 16 mg. per kg.) the rate was 210 at the beginning of the injection, 188 three minutes later, and 208 twenty minutes after that.

Fig. 6 illustrates a record obtained with a membrane manometer showing the slowing of the heart and fall of blood pressure in a decapitate cat following an injection of 17 mg. per kg.

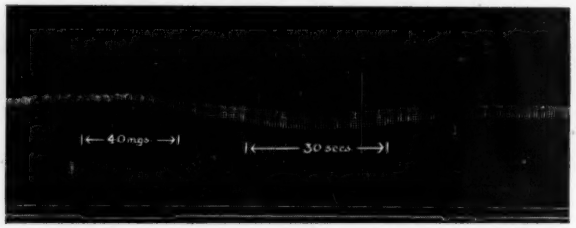

Fig. 6.—Decapitate cat. Blood pressure recorded by membrane manometer. 40 mgm. proactinomycin (17 mg. per kg.) intravenously. Note fall of blood pressure and slowing of heart rate.

Action on other systems.

Effect on vagus and splanchnic nerves.—In the course of these experiments it was found that the administration of proactinomycin (17 mg. per kg.) to a cat under chloralose did not affect the response of the heart to vagal stimulation nor the rise in blood pressure on stimulating the L. splanchnic nerve (Fig. 7). Thus there was no evidence of an atropine-like or ergotamine-like action.

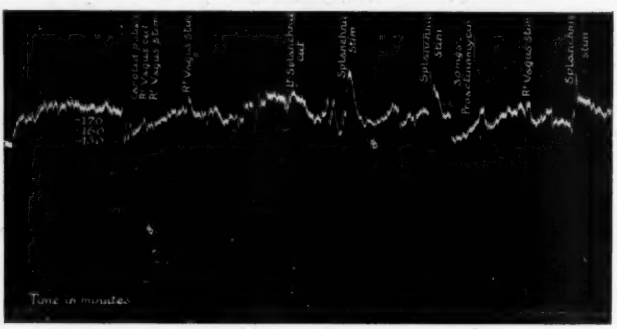

Fig. 7.—Blood-pressure record of cat under chloralose. Stimulation of right vagus and left splanchnic beginning 22 minutes after 50 mg. proactinomycin (17 mg. per kg.) intravenously. Both effects still present after further 30 mg. of drug.

Effect on respiration.—There was no significant alteration in the respiration rate during or after the administration of proactinomycin to the cats.

Other effects.—No pharmacological effects were noted in other systems in these cats. There was no activity or apparent paralysis of voluntary muscle and no evidence of stimulation of smooth muscle. There was no excessive salivation. A steady flow of bile and urine was seen in those animals in which the common bile duct and the urethra were cannulated. Urine was freely secreted in mice injected with proactinomycin in another connection.

Action on smooth muscle.—For these experiments the uterus of the virgin guinea-pig was used. One part in 25,000 of proactinomycin was capable of lessening, but not entirely abolishing, the rhythmic contractions, and full contraction was

FIG. 8.—Guinea-pig uterus. Shows reduction of rhythmic contractions by 1/25,000 proactinomycin with restoration on washing with Ringer's solution.

restored on washing the preparation with Ringer's solution (Fig. 8). In 1 : 5000 the contractions were almost entirely abolished, but nevertheless the uterine muscle responded with a normal contraction at once on the addition of histamine (Fig. 9).

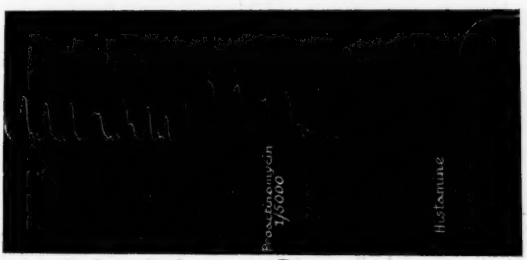

FIG. 9.—Guinea-pig uterus. Shows inhibition of contraction by 1/5000 proactinomycin, but retention of contractile power to histamine.

Toxicity.

To tissue cells.—The previous report on the toxicity of proactinomycin to human leucocytes *in vitro*, determined by the method of Abraham *et al.* (1941), was confirmed. A 1 : 1000 solution killed the leucocytes in about half an hour. In successive dilutions there was progressively less effect till at 1 : 16,000 the preparation was indistinguishable from the controls.

The effect of local application to tissues was examined also by placing a solution of proactinomycin in direct contact with the central nervous system *in vivo*. In a rabbit anaesthetized with ether a hypodermic needle was passed into the cisterna magna and 0·75 c.c. of a 1 : 1000 solution of proactinomycin in saline was injected. No phenomena attributable to the proactinomycin were seen during or after recovery and the rabbit remained well. At 24 hours a sample of cerebrospinal fluid was withdrawn for testing by plate and cylinder, but by this not very delicate method no activity could be detected.

Acute toxicity to mice.—1 mg. intravenously produced signs of sickness though the mice recovered within half an hour. 2 mg. killed immediately. By stomach tube 5 mg. was well tolerated, but after 10 mg. the mice were usually sick for some time, though they recovered. A dose of 14 mg. killed in one and a half hours.

Chronic toxicity to mice.—Proactinomycin was given by stomach tube to 20 g. mice in doses ranging from 0·625 to 5 mg. twice daily for 5 days, and the animals were

killed on the 6th day or later. No organ showed any obvious abnormality except the liver, which histologically showed evidence of some damage. Many of the cells contained considerable amounts of fat, which in ordinary paraffin sections gave them a highly vacuolated appearance. This liver damage was not sufficient to produce any definite deviation from the normal in the appearance or behaviour of the mice, or in their subsequent survival. In some of the animals killed several weeks after the injections there was histological evidence of recovery.

Absorption, excretion and distribution in the body.

It was clear that proactinomycin was absorbed from the stomach, as after a sufficiently large dose by stomach tube mice became ill or died, the fatal dose being about 10 times greater than by the intravenous route.

Three mice weighing 25 g. were given 10 mg. by stomach tube and were killed from 5 to 5½ hours later, at which time they showed signs of sickness. Samples of blood serum and urine, washings from the lumen of the stomach and small intestine and a saline extract of liver were tested for the presence of proactinomycin by the plate and cylinder method, the plates being sown thinly with *Staphylococcus aureus*.

The results, which were qualitative only, were entirely consistent in the three animals. The urine contained substantial amounts of active substance, but no activity was detectable in the blood serum. Liver extract produced inhibition, and there was a considerable amount of active substance still left in the lumen of the gastro-intestinal tract.

In several of the cats used for blood-pressure observations, which received intravenously from 12 to 24 mg. per kg. of proactinomycin, the cerebrospinal fluid, pericardial fluid or other fluids or tissue extracts were sampled, and in three cats bile and urine were collected half-hourly or hourly throughout the experiment. All tests were made by plate and cylinder. Cerebrospinal fluid showed a trace of activity once (out of three samples) and pericardial fluid (2 samples), saliva (1 sample) and blood (1 sample) no activity. Extracts of spleen and lymph gland were negative (one sample of each). As in mice the kidneys effected concentration and the urine showed activity throughout the experiments, the longest period after injection being 5¼ hours. Similarly there was concentration by the liver with prolonged secretion into the bile.

The blood of another series of mice was tested by Dr. N. G. Heatley with the modified slide cell test (Garrod and Heatley, 1944), in which *Staph. aureus* is inoculated directly into the blood serum—a more sensitive method than plate and cylinder. Five mice were killed at 1, 1, 2, 3 and 4 hours respectively after receiving 5 mg. by stomach tube, and another 5 mice at ¼, ½, 1, 2 and 3 hours after 1 mg. intravenously. It was a considerable surprise to find that at no time was there any inhibition of the growth of staphylococci by the serum.

Partition in blood in vitro.—The partition between plasma and blood cells was studied first with rabbit and then with human blood. Citrated blood containing 1 : 1000 proactinomycin was rotated at 37° C. for 4 hours, after which samples of plasma, lysed red blood cells and lysed whole blood were titrated for antibacterial activity by the dilution method, using *Staphylococcus aureus* as the test organism.

The results showed that the proactinomycin was distributed evenly between the elements of the blood, but that there was a loss of activity of about 50 per cent. in all samples compared with an aqueous solution.

In the experiment with human blood the leucocytes were separated and were lysed by heating and shaking with beads, but as some plasma was mixed with them

the titration could only be expected to show a difference in concentration several times greater than in the other elements of the blood. No such difference was apparent.

2. *Antibacterial Activity.*

Action against various species and effect of inoculum size.

As proactinomycin is highly soluble in water its antibacterial activity is readily tested by the serial dilution method, as Gardner and Chain (1942) showed. Many titrations of this sort were carried out. Different preparations of proactinomycin were in use at different times, the proportion of impurities fluctuating probably between about 10 and 30 per cent.

All titrations except that of the tubercle bacillus were done in heart extract broth which had been tested for its ability to support growth from a very small inoculum. For streptococci, pneumococci, neisseria and corynebacteria 10 per cent. of horse serum was included. (The presence of 10 per cent. of serum does not affect the titre (Gardner and Chain, 1942).) Tubes containing serial dilutions in 5 c.c. of broth were inoculated uniformly from a Dreyer's pipette, and the results were read after overnight incubation at 37° C. The tubercle bacillus was tested by growing film cultures of

TABLE I.—*Antibacterial Activity of Proactinomycin by the Serial Dilution Method. Titration against Various Species.*

Bacterium.	Number of experiments.	Dilution of culture used for inoculum.	Highest dilution of proactinomycin giving inhibition.		Lowest dilution giving no inhibition.
			Complete.	Partial.	
Str. pyogenes, Richards strain	1	*nil* (2 drops)	400,000	800,000	1,600,000
	2	*nil*	320,000	1,280,000	—
	4	1 : 10	160,000 to 640,000	640,000 to 2,560,000	1,280,000 to 5,120,000
Str. viridans (from subacute bacterial endocarditis)	1	*nil*	256,000	1,024,000	—
Str. pneumoniae, Type I	1	,, (2 drops)	200,000	1,600,000	3,200,000
N. meningitidis	1	*nil* (5 drops)	800,000	3,200,000	6,400,000
Staph. aureus, N.C.T.C., No. 6571	2	*nil*	32,000	128,000	256,000
			64,000	256,000	512,000
	7	1 : 1000	128,000 to 640,000	256,000 to 1,280,000	512,000 to 2,500,000
	2	1 : 1,000,000	256,000	512,000	1,024,000
			512,000	1,024,000	2,048,000
C. diphtheriae	1	*nil*	512,000	..	1,024,000
	1	1 : 1000	2,048,000	4,096,000	8,192,000
C. hofmannii	1	*nil*	512,000	..	1,024,000
	1	1 : 1000	2,048,000	..	4,096,000
B. anthracis	1	*nil*	256,000	..	512,000
	1	1 : 1000	1,024,000	..	—
Salm. enteritidis (Gaertner)	2	1 : 1000	32,000	64,000	128,000
			160,000	320,000	640,000
Myco. tuberculosis, human	2	:	—	10,000	100,000
Salm. typhi	1	1 : 1000	—	—	1000
Bact. coli	1	1 : 1000	—	—	1000
Proteus	1	1 : 1000	—	—	1000
Ps. pyocyanea	1	1 : 1000	—	—	1000

The inoculum was 1 drop except where noted otherwise.

A dash indicates that the figure was outside the range of the titration, and a blank that it fell between two dilution tubes which were next to each other in the series.

TABLE II.—*Antibacterial Activity of Proactinomycin by the Serial Dilution Method. Effect of Inoculum Size on Titration against* Staph. aureus.

Experiment 1.

Concentration of proactinomycin.	Dilution of inoculum 1 in— 1.		1000.		1,000,000.	
1 : 32,000	−	−	−	−	−	−
1 : 64,000	+	+	−	−	−	−
1 : 128,000	+	+	−	−	−	−
1 : 256,000	++	+	−	+	−	−
1 : 512,000	++	++	+	+	+	+
1 : 1,024,000	++	++	++	++	++	++

Experiment 2.

	Dilution of inoculum 1 in— 1.	10.	100.	1000.	10,000.	100,000.	1,000,000.
1 : 64,000	−	−	−	−	−	−	−
1 : 128,000	+	+	−	−	−	−	−
1 : 256,000	+	+	+	+	−	−	−
1 : 512,000	++	+	++	++	+	+	−
1 : 1,024,000	++	++	++	++	++	++	+
1 : 2,048,000	++	++	++	++	++	++	++

− = No growth ; + = partial growth ; ++ = growth as in control.

sputum (Pryce, 1941; Muller, 1944) in a medium consisting of 10 per cent. packed human red cells from citrated blood with 90 per cent. distilled water to which proactinomycin had been added. To allow for inactivation of proactinomycin by the blood during the incubation period of one week at 37° C., samples were tested and fresh solution added as required. The final concentration in each bottle, as shown by assay at the end of the experiment, was at least that stated. Only one strain of each organism was used except with the tubercle bacillus, which came from a different patient in each experiment.

The results are set out in Tables I and II ; the former includes the limits of variation when an organism was tested more than once. It is clear that the size of the inoculum has a very considerable effect on the dilution at which inhibition of growth is effected. In spite of the difficulty of fixing an inhibition figure precisely for each species, Table I falls into two parts, and confirms that proactinomycin is an antibiotic which acts strongly against cocci and Gram-positive bacilli and weakly against the tubercle bacillus and Gram-negative bacilli, with *Salm. enteritidis* (Gaertner) occupying, as with penicillin (Abraham *et al.*, 1941) and helvolic acid (Chain *et al.*, 1943), an intermediate position.

An investigation of the activity of proactinomycin against bacteria pathogenic to plants will appear elsewhere (Gilliver, 1945).

Inhibition of antibacterial action by serum and blood.

Gardner and Chain (1942) noted that the presence of 10 per cent. of serum did not prevent a high titre of antibacterial activity against streptococci and neisseria, but they did not test the effect of higher concentrations. The effect of 50 per cent. of

serum, blood and lysed red cells was examined by the plate and cylinder method. Proactinomycin at 1 : 5000 in the various mixtures was incubated with rotation at 37° C. for 5 hours and then placed in cylinders on *Staph. aureus* plates and incubated overnight. The serum had no detectable effect, but whole blood and lysed red cells somewhat reduced the size of the ring.

As shown by the serial dilution method, the effect of incubating for 4 hours at a concentration of 1 in 1000 in undiluted whole blood was to reduce the activity 50 per cent.

Development of resistance by bacteria in vivo.

During the course of the mouse protection experiments *Strept. pyogenes* was exposed to the influence of proactinomycin *in vivo* for various lengths of time, and when a death occurred among these mice the organism was recovered and its sensitivity determined. The treatment given before death varied from three doses of 0·1 mg. of proactinomycin intraperitoneally in 24 hours to 10 doses of 5 mg. by mouth in 4½ days. Titrations were done in all on 12 recovered strains, in 9 cases (including that which had had the largest total dose) with the primary culture. No variation from the original sensitivity had occurred in any strain.

Effect on penicillin-resistant bacteria.

Proactinomycin was titrated in parallel against four strains of staphylococcus, two showing natural resistance to penicillin, one made highly resistant to penicillin *in vitro* and one sensitive to penicillin. The titre of proactinomycin was the same for all these strains, showing that there was no cross resistance.

Proactinomycin and helvolic acid.

It was not possible to demonstrate any synergism in the action of proactinomycin and helvolic acid *in vitro* against *Staph. aureus* or *Salm. enteritidis* (Gaertner).

Mouse protection tests.—The details of these experiments are set out in Table III. They were carried out on white mice weighing 20 g., the infecting organism being in all experiments a Richards strain of *Str. pyogenes.** In the first two experiments a small dose of streptococci was used, though one which would certainly kill all mice. In the third, since smaller doses of proactinomycin were being tried, the infecting dose was reduced, and in this experiment 2 out of the 10 controls did not die.

In the first experiment the 10 controls all died within 48 hours, while 2 out of 5 treated mice survived and the life of the others was prolonged—this group had a shorter period of therapy than those in the succeeding experiments. In the second experiment the 10 controls died within 52 hours and 6 out of the 10 treated mice survived, the life of the rest again being prolonged. In the third 2 out of the 10 control mice survived and 1 out of the 10 treated intraperitoneally, while 13 out of 15 of the mice treated by the oral route survived. The streptococcus was recovered from all mice which died within the period of the experiment (14 days), with the exception of one which was not examined.

It is clear that proactinomycin had a definite chemotherapeutic effect in these experimental streptococcal infections in mice. The dose of 5 mg. orally is near the toxic limit, but it is very surprising that a dose of as little as 0·625 mg., given twice daily, was, so far as can be judged on the small number of animals, as good as a dose of 5 mg. twice daily, especially when it is recalled that after doses of even 5 mg. orally,

* Strain C.N. 10 of the Wellcome Physiological Research Laboratories, kindly provided by Dr. J. W. Trevan.

TABLE III.—*Mouse Protection Tests with Proactinomycin.*

Proactinomycin in solution was given 12-hourly, beginning 1 hour after intraperitoneal infection with *Str. pyogenes*. Weight of mice = 20 g.

Experiment number.	Infecting dose of 16–20 hour culture.		Treatment.						Number of mice.	Number of survivors at end of day.													
			Single dose. mg.	Total dose. mg.	Number of doses.	Interval hours.	Duration. Days.	Route.		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.
1	0·5 c.c. of 1 : 1000	Control	..	..	..	..	..	..	10	9	0	..	..	..	..	..	..	..	..	..	..	..	0
		Treated	5	30	6	12	3*	Oral	5	..	..	..	..	4	3	..	..	..	..	..	..	..	2
2	0·5 c.c. of 1 : 500†	Control	..	..	..	..	..	..	10	6	1	0	..	..	..	..	..	..	..	..	..	..	0
		Treated	5	50	10	12	4½	Oral	5	..	..	..	4	..	..	..	3	..	..	..	..	..	3
			2·5	25	10	12	4½	„	5	..	..	..	..	..	..	4	..	..	..	..	..	3	3
3	0·5 c.c. of 1 : 1000	Control	..	..	..	..	..	..	10	6	2	..	..	..	..	..	..	..	..	..	..	..	2
		Treated	0·1	1·0	10	12	4½	Intraperitoneal	10	..	6	3	2	..	1	..	..	..	..	..	..	..	1
			2·5	25	10	12	4½	Oral	5	..	..	..	..	..	..	..	..	4	..	..	..	..	4
			1·25	12·5	10	12	4½	„	5	..	4	..	..	..	..	..	..	..	..	..	..	..	4
			0·625	6·25	10	12	4½	„	5	..	..	..	..	..	..	..	..	..	..	..	..	..	5

* 24 hours between last two doses.
† Virulence fell between Experiments 1 and 2.

no antibacterial activity (against the staphylococcus) could at any time be detected in the serum. It is remarkable also that intraperitoneal injections of the solution had practically no apparent effect on the course of the infection.

3. *Activity Against Protozoa.*

Trypanosomes.

The experiments were carried out with *T. equiperdum.** An *in vitro* test was done by inoculating 10 c.mm. of citrated mouse blood containing a total of about 1000 trypanosomes into tubes containing 1 c.c. of tenfold serial dilutions of proactinomycin in Ringer's solution with 50 per cent. rabbit serum. The concentrations were from 1 : 10,000 upwards. The preparations were examined six times from the 3rd to the 12th hour. At no time were trypanosomes seen in the tube containing 1 : 10,000 proactinomycin, but in 1 : 100,000 and higher dilutions they were as plentiful throughout as in the controls.

This was followed up by an *in vivo* test in which groups of infected mice were treated with large doses intravenously and by stomach tube, but the number of trypanosomes in the blood was not reduced, and the mice sickened and died as quickly as the control animals.

Amoebae.

Following the work of Laidlaw, Dobell and Bishop (1928) on emetine, the test was carried out *in vitro* in a liquid medium consisting of horse-serum diluted 1 : 8 with Ringer's solution, with a little rice-starch added. *E. histolytica*† was inoculated into tubes containing tenfold serial dilutions of proactinomycin in this medium, the strongest concentration being 1 : 5000. This value was unchanged when the medium was assayed at the end of the 5 days of incubation. Throughout this period the amoebae remained plentiful and active in all concentrations of proactinomycin; indeed there was an "impression," which was not seriously pursued, of more rapid movement in the presence of proactinomycin. The accompanying bacteria appeared to be unaffected by the proactinomycin.

Leishmania.

Proactinomycin to a final concentration of 1 in 5000, 50,000 and 500,000 was added to N.N.N. medium before sloping and *L. donovani*† was inoculated into the water of condensation in six tubes at each dilution. After 4 days' incubation at 24° C. active Leishmania were present in all the tubes at 1 : 50,000, but only in 1 out of the 6 at 1 : 5000. The water of condensation from the latter was withdrawn for testing, though accurate assay was not possible on these small amounts of fluid. There was some variation between the tubes, but the average concentration of proactinomycin in the fluids was in the neighbourhood of 1 : 10,000, and certainly not below 1 : 20,000. It appears that, as with the trypanosome, the inhibitory figure is of the order of 1 : 10,000.

4. *Action against Fungi.*‡

In all 55 species of fugus and 1 yeast were tested, comprising saprophytic fungi and fungi pathogenic to man and plants. No species was inhibited by proactinomycin at 1 : 5000, the strongest solution tested.

* Provided by the Department of Pharmacology, University of Oxford.

† Provided by Dr. C. A. Hoare, of the Wellcome Laboratories of Tropical Medicine, to whom we are also indebted for advice on methods of culture.

‡ The fungi pathogenic to plants were tested by K. Gilliver.

5. *Proactinomycin for Differential Cultivation.**

Some experiments were done to show whether the addition of proactinomycin to a solid medium would make it easy to isolate insensitive species of bacteria from mixtures of sensitive and insensitive species.

(1) On boiled-blood-agar containing proactinomycin in a concentration of 1 in 40,000 a plating was made of 1 loopful of a feebly turbid suspension of *H. influenzae* with 1 c.c. of a 24 hours' broth culture of *Staph. aureus* and 1 c.c. of a 24 hours' serum-broth culture of *Str. pyogenes*. After incubation for 48 hours at 37° C. there was a rich growth of *H. influenzae*, but no growth of cocci. A control plating on plain boiled-blood-agar showed a profuse growth of staphylococci and streptococci, with no recognizable *Haemophilus*.

(2) An experiment done on the same principles showed that from a mixture of *Bact. coli* with staphylococci and streptococci the first of the three was the only one to develop in a plating on agar containing 1 in 40,000 of proactinomycin.

The relative stability of proactinomycin suggests that it might be more suitable than penicillin as an ingredient of culture media. The range of insensitive organisms for whose isolation it might be useful is, so far as tested, similar to that for penicillin.

DISCUSSION AND SUMMARY.

As a result of the foregoing investigations it can be seen that proactinomycin possesses chemotherapeutic power in that when given to mice by mouth it can confer a considerable degree of protection against intraperitoneal infection with haemolytic streptococci. This effect takes on additional interest from the fact that even with a large dose it has not been possible to detect in the blood any antibacterial effect (using the staphylococcus, which is quite sensitive, as the test organism). Nevertheless, not only this large dose, but a dose one-eighth the size, had a chemotherapeutic effect.

Like other relatively innocuous antibiotics, such as penicillin and helvolic acid, proactinomycin is excreted in the urine and bile. It is absorbed from the alimentary canal. In sufficiently large doses it stimulates the vaso-motor centre and decreases the rate and force of the heart-beat in cats, and inhibits the contraction of the guinea-pig uterus. Repeated injections cause fatty changes in the livers of mice.

The question naturally arises whether this substance can be of interest in the treatment of disease in man. It is undoubtedly much more toxic than penicillin or streptomycin (Robinson, Smith and Graessle, 1944), and where these can be made effective there would be no indication for the use of the more toxic substance. Nevertheless there is increasing evidence that resistance to penicillin can be acquired by certain organisms, particularly the staphylococcus, and this phenomenon may well be true of all chemotherapeutic agents. It is therefore well to take note of substances such as this, to which a penicillin-resistant staphylococcus is fully sensitive, which might, at any rate in local application, provide a possible alternative.

This work is also of interest in that it shows that yet another substance from the Actinomyces and Proactinomyces group of organisms has chemotherapeutic properties, though it is not comparable to the others, particularly streptomycin, in this respect.

We are indebted for technical assistance to the Misses M. Lancaster, P. McKegney and J. Stewart, who prepared the material, to Miss P. Morris, who carried out many of the bacteriological titrations, and to Mr. J. Kent, who assisted with the animal work.

* By Prof. A. D. Gardner.

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THE EFFECT OF PROACTINOMYCIN ON *STAPHYLOCOCCUS AUREUS* IN HEART BROTH.

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THE antibiotic proactinomycin was first described by Gardner and Chain (1942). Its biological properties have now been investigated in detail by Florey, Jennings and Sanders (1945), who observed that the titre of its antibacterial effect in the serial dilution test depended, *inter alia*, on the size of the bacterial inoculum. The present paper records a quantitative investigation of the effect of proactinomycin on *Staphylococcus aureus* in heart broth. In low concentrations the antibiotic was found mainly to lengthen the lag period of the staphylococci, but as the concentration was increased above a limiting value its action rapidly became bactericidal.

METHOD.

The experimental method was the same as that used for investigating the effect of another antibiotic (mycophenolic acid) on the staphylococcus (Abraham, 1945). The staphylococcus (H. Strain, N.C.T.C. No. 6571) was grown at 37° C. in heart extract broth of pH 7·4 aerated by a slow stream of air. A single batch of heart broth was used for all the measurements. The inoculum consisted of three to five drops of a suitably diluted 16-hour aerated culture.

Total counts were made in a Neubauer haemocytometer which could be used for counting suspensions containing more than about $5{\cdot}0 \times 10^6$ cells per ml.

Viable counts were made by spreading two drops of the diluted culture on the surface of nutrient agar plates.

Under the conditions used the staphylococcus normally grew in the form of discrete cells. In the presence of proactinomycin the organisms showed a tendency to grow in pairs, but larger aggregates of cells were rarely seen.

The specimen of proactinomycin used was of high activity, but was probably not chemically pure. The dilutions in which it was used have thus no absolute significance.

The growth curves show the variation of the logarithm of the number of bacteria per ml. with the time.

The usual growth curve of a bacterial culture consists of a lag period followed by a period in which the organisms multiply logarithmically. During logarithmic growth the time t taken for the number of organisms to increase from n_1 to n_2 is given by the equation $n_2 = n_1.e^{kt}$ or $2{\cdot}303 \log_{10} n_2/n_1 = k.t$, where k is a growth constant.

The transition from the lag phase to the phase of logarithmic growth is often by no means sharp. The length of the lag L is defined here formally by the expression $n = n_0.e^{k(t-L)}$, where n_0 is the number of organisms in the inoculum and n the number present after a time, t, at which logarithmic multiplication is taking place. L is thus determined by extrapolating backwards the straight line which represents the variation of log n with t during active multiplication : when log n equals log n_0 the corresponding value of t equals L.

The time taken for the cells to double in numbers during active growth is called the mean generation time (m.g.t.). It follows that $2{\cdot}303 \log_{10}2 = k \times$ m.g.t. The value of k and hence that of the m.g.t. is obtained from the slope of the growth curve, since $2{\cdot}303\ d \log_{10}n/dt = k$.

Normal growth in heart broth.

In the sample of heart broth used for these experiments the staphylococcus multiplied with a m.g.t. of 28 minutes after a lag of about 90 minutes (curve 1, Fig. 1). The rate of multiplication diminished rapidly when the number of cells approached 900×10^6 per ml., which was taken as the size of the stationary population.

Earlier measurements of growth in heart broth had shown that neither the length of the lag nor the rate of division altered significantly when the inoculum size was varied from nearly 10×10^6 to $0{\cdot}01 \times 10^6$ cells per ml. (Abraham, 1945).

Growth in the presence of 1 *in* 500,000 *proactinomycin.*

Growth in the presence of 1 in $0{\cdot}5 \times 10^6$ proactinomycin from inocula of different sizes is shown by curves 2 to 4 in Fig. 1. At this concentration the antibiotic lengthened the lag period and decreased the rate of division, but had little effect on the size of the stationary population.

The increase in the length of the lag period became much greater as the inoculum size is reduced. The figures are given in Table I.

TABLE I.

Inoculum (cells per ml.).	Lag (minutes).	m.g.t. (minutes).
$6{\cdot}40 \times 10^6$	200	60
$0{\cdot}30 \times 10^6$	330	60
$0{\cdot}03 \times 10^6$	730	65

Some hours after the two larger inocula had multiplied to their stationary populations, cells from these cultures ($n_0 = 6{\cdot}0 \times 10^6$) were again inoculated into heart broth containing 1 in $0{\cdot}5 \times 10^6$ proactinomycin. The resulting growth curves were not detectably different from the primary curves. There was thus no rapid adaptation to growth in the presence of proactinomycin comparable to that which occurred with mycophenolic acid.

Effect of filtrates from young cultures on growth in the presence of proactinomycin.

Since in the presence of the antibiotic the lag was shortened by increasing the number of cells in the inoculum, it was important to find out the effect on the lag phase of filtrates from actively growing cultures. Two different experiments were carried out :

(1) An inoculum of 5×10^6 cells per ml. was allowed to grow to 50×10^6 in a sample of heart broth. The organisms were then centrifuged, and the supernatant solution was sterilized by filtration through a gradacol membrane.

Growth was then compared, from inocula of $0{\cdot}1 \times 10^6$, in heart broth and in the filtrate, after 1 in $0{\cdot}5 \times 10^6$ proactinomycin had been added to both media. The results are shown in Fig. 2. The filtrate had no detectable effect on the m.g.t., but it reduced the lag period from 600 to 180 minutes.

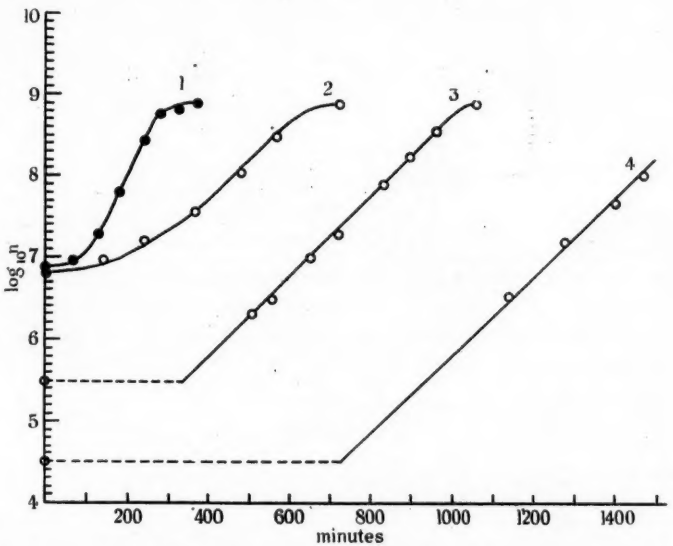

FIG. 1.—Effect of inoculum size on growth in the presence of proactinomycin. Curve 1 : Growth in normal heart broth. Curves 2, 3, and 4 : growths in the presence of 1 in 500,000 proactinomycin.

(2) A second filtrate was prepared by allowing an inoculum of 5×10^6 cells per ml. to grow to 50×10^6 in heart broth containing 1 in $0{\cdot}5 \times 10^6$ proactinomycin. Growth from a small inoculum in this filtrate was then compared with that in heart broth to which the same amount of proactinomycin had been added. The results were very similar to those obtained in (1). The filtrate caused a great reduction in the length of the lag period.

Effect of concentration of proactinomycin.

(*a*) In Fig. 3 curves 2, 3 and 4 show the effect on growth from fairly large inocula ($n_0 = 5{\cdot}0 \times 10^6$) of increasing the concentration of proactinomycin from 1 in 10^6 to 1 in $0{\cdot}25 \times 10^6$. The data are given in Table II. Over this range the length of the lag period increased considerably with the concentration of the antibiotic, starting from

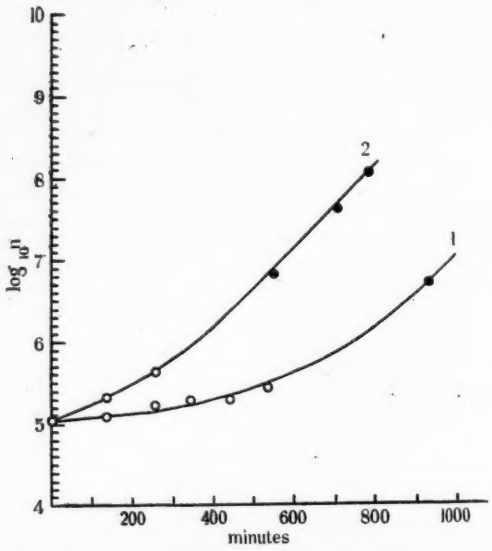

FIG. 2.—Effect of filtrate on growth in the presence of proactinomycin. Curve 1 : growth in heart broth containing 1 in 500,000 proactinomycin. Curve 2 : growth in heart broth filtrate containing 1 in 500,000 proactinomycin. —●—●— Total counts. —○—○— Viable counts.

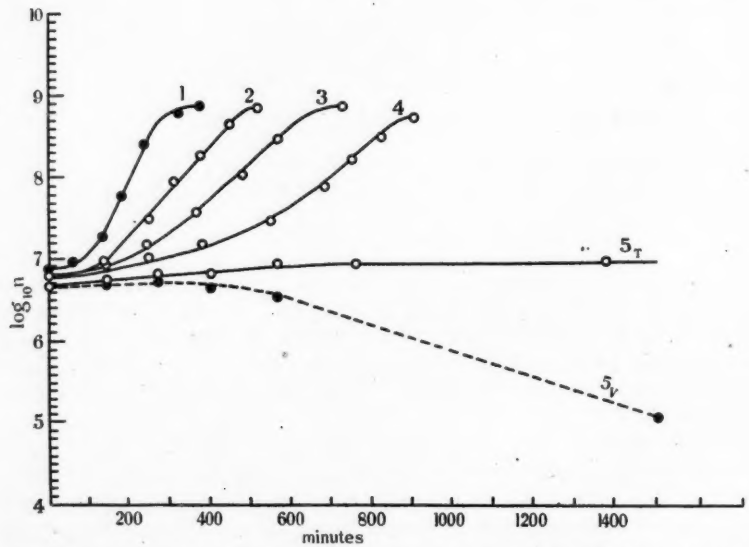

FIG. 3.—Effect of concentration of proactinomycin. Curve 1 : growth in normal heart broth. Curves 2–5 : growth in increasing concentrations of proactinomycin.

a value at the lowest concentration, which was not much greater than that of a control culture.

The effect of concentration on the rate of division during the logarithmic phase was quantitatively quite different. At the lowest concentration the m.g.t. was nearly twice that of a control culture, but a fourfold increase in the amount of proactinomycin

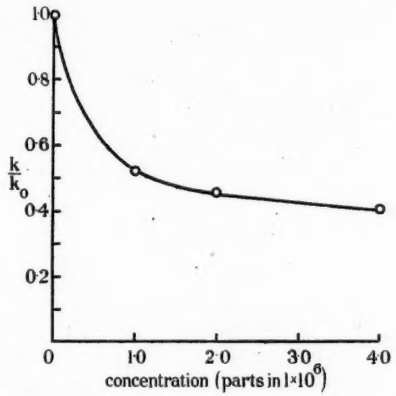

FIG. 4.—Variation in the growth constant with the concentration of proactinomycin.

led to only a relatively small further decrease in the rate of division. This is shown in Fig. 4, in which k/k_0 is plotted against the concentration. k_0 is the growth constant of a normal culture and k that of a culture growing in the presence of a given concentration of the antibiotic. The values of k_0 or k are inversely proportional to the m.g.ts.

TABLE II.

Concentration of proactinomycin.	Lag (minutes).	m.g.t. (minutes).
1 in 10^6	110	52·8
1 in $0{\cdot}5 \times 10^6$	200	60·5
1 in $0{\cdot}25 \times 10^6$	400	68·0

(*b*) When the concentration of proactinomycin was increased to 1 in $0{\cdot}1 \times 10^6$ a more profound effect was exerted on the staphylococcus. This is shown by curves 5_T and 5_V (Fig. 3), which represent total and viable counts respectively. A very slow rate of division took place until the total number of organisms had approximately doubled in 800 minutes, but after this no further cell division occurred. During the first 300 minutes almost all the organisms were viable, but subsequently the cells apparently began to die,* and after 1500 minutes only about 2 per cent. remained viable.

When 1 in $0{\cdot}1 \times 10^6$ proactinomycin was added to a culture in the phase of logarithmic multiplication ($n = 10 \times 10^6$) growth at first continued at a reduced rate.

* It is evident from a number of investigations (e.g. Valko and Dubois, 1944) that the failure of cells to multiply into colonies on plating out is not a sure criterion that they are dead. In the present instance the term " non viable " is used with reference to the conditions employed. Such non viable cells must have undergone some change, and it would appear reasonable to refer to them as " dead " until evidence is obtained to the contrary.

The number of viable organisms, which remained very close to the total number of organisms, doubled in about 140 minutes. After this the total number of organisms increased very slowly for 300 minutes and then remained constant, but the number of viable organisms diminished until after 1200 minutes these represented about 20 per cent. of the total.

DISCUSSION.

The lag phase.

The most striking effect of low concentrations of proactinomycin on the growth of the Oxford staphylococcus is the lengthening of the lag period to an extent which depends, in the medium used here, on the size of the inoculum. An increase in the lag with a decrease in the inoculum size clearly suggests that substances which are concerned with the ending of the lag may be lost by diffusion from the cells.

Many instances are recorded in the literature of bacteria growing well, on liquid or solid media, from a large inoculum, but either failing to grow or only doing so after a prolonged lag period when the inoculum was small. Cohen, Snyder and Mueller (1941) investigated the growth factors involved in this phenomenon in the case of a diphtheria bacillus, but in most cases little was done beyond recording the observations. While investigating the growth of *Bacterium lactis aerogenes* in an artificial medium, however, Lodge and Hinshelwood (1943) found that the length of the lag period was considerable when the inoculum was taken from very young cultures, then fell to a minimum, and finally became greater again as the age of the culture increased. The first or "early lag" was shortened by an increase in the size of the inoculum, or by the addition of filtrate from actively-growing cultures at the stage of minimum lag. Davies, Pryce and Hinshelwood (1944) found that early lag was greatly increased by proflavine and methylene blue, and that this effect of the drugs was antagonized by filtrate from a young culture. They concluded that a diffusible substance was concerned with the ending of early lag, and that the synthesis or utilization of this substance was affected by the drugs.

Abraham (1945) found that the length of the lag period of the Oxford staphylococcus grown in heart broth did not change significantly with considerable variations in inoculum size, but that when mycophenolic acid was added to the medium the lag increased greatly as the size of the inoculum was made smaller, and that the prolonged lag period with small inocula was abolished by filtrates from young cultures.

The effect of proactinomycin on the lag phase of the staphylococcus, which is similar to the effect of mycophenolic acid, is readily explained by assuming that one of the primary effects of the antibiotic is an interference with the production or use by the cells of a diffusible growth intermediate. An example of an interference with the use of such a substance would be the alteration of the dissociation constant of a coenzyme and its protein carrier. Subsequent experiments, however, have shown that the matter is complicated by the fact that the activity of proactinomycin is measurably decreased by relatively small increases in the acidity of the medium (Abraham and Duthie, 1945). Acids produced by the organisms during growth have thus to be considered as diffusible substances which may contribute to a shortening of the lag. The pH of filtrates from young cultures was about 0·1 unit lower than the pH of the original medium. This increase in acidity is responsible for part, but not all, of the decrease in the length of the lag which is found in the presence of filtrate.

Phase of logarithmic growth.

The effect of proactinomycin on the phase of active division is quantitatively quite different from its effect on the lag phase. In high dilutions the antibiotic causes

a considerable increase in the m.g.t., but as its concentration is raised the further decrease in the rate of division is relatively quite small. This tendency for the rate of division to fall to a limiting value in the presence of increasing concentrations of a drug has been found in other cases (Davies and Hinshelwood, 1943 ; Abraham, 1945). The variation of the growth constant k with the concentration of the antibiotic C may be expressed by an equation of the type $k = k_0\left(1 - \frac{aC}{1 + bC}\right)$, where a and b are constants. $\frac{k_0 aC}{1+bC}$ is an expression for the amount of a substance at concentration C, which is adsorbed on a surface if a fraction $(1 - a/b)$ of the surface is non adsorbing. The limiting value of k/k_0, which is also $(1 - a/b)$, might similarly be equated with a fraction of the mechanism controlling the rate of logarithmic growth which is relatively insensitive to the antibiotic.

Bactericidal action of proactinomycin.

When the concentration of proactinomycin is increased above a limiting value the behaviour of the staphylococcus changes fairly abruptly, as though fresh centres in the cell were being affected. Instead of the cells dividing fairly rapidly once logarithmic multiplication has set in, growth stops and the cells apparently begin to die soon after an incipient transition from the lag phase to the logarithmic phase is discernible. When the antibiotic is added during active multiplication a decrease in the number of viable organisms begins after about one cell division. Qualitatively there is a superficial resemblance, at least, between this behaviour and the behaviour of the staphylococcus towards penicillin. In both cases the lethal effect is not immediate, but follows after a period of active growth or preparation for active growth. The mechanisms of killing thus appear to differ from those concerned when the cells die logarithmically *ab initio*. The facts suggest that the interference by the drugs with a cell reaction accompanying growth results in the building up of products inimical to the life of the cell.

The effect of inoculum size in the serial dilution test for proactinomycin activity.

If a number of tubes containing serial dilutions of an antibacterial substance and inoculated with a test organism are read *after a limited time*, it is clear that decreasing the inoculum size may result in the absence of growth at a somewhat higher dilution merely because the organisms must then go through more divisions before growth is visible. Thus, suppose the organisms grow from two inocula n_1 and n_2 in the presence of a given concentration of the antibiotic, as shown in Fig. 5, and that n_v is the number of organisms below which growth is not visible. Between times t_1 and t_2 the first culture is fully grown, while no growth is visible in the second. $(t_2 - t_1)$ becomes larger the slower the rate of growth. In this way a thousandfold decrease in the inoculum size might easily lead to a difference of one tube in a two-fold dilution series. This difference would disappear if incubation were continued for a sufficient time.

When the activity of proactinomycin is measured against the staphylococcus two factors may contribute towards an extension of this difference. These are the increase of the lag period as the inoculum size is reduced, and the small variation, over a limited range, in the rate of growth with changes in the concentration of the antibiotic. A comparison of curve 4, Fig. 1, and curve 4, Fig. 3, shows that between 15 and 20 hours after inoculation full growth had occurred from an inoculum of 5×10^6 cells per ml. in 1 in 250,000 proactinomycin, but no growth was visible from an inoculum of $0{\cdot}03 \times 10^6$ in 1 in 500,000 proactinomycin ; this would correspond to a difference of two tubes

in a two-fold serial dilution test. Since, however, on increasing the concentration of proactinomycin to 1 in 100,000 its effect became bactericidal and no significant growth

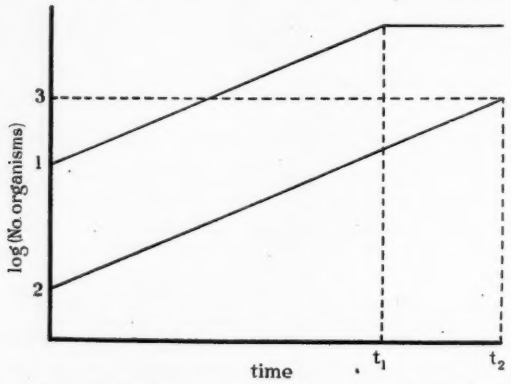

FIG. 5.—Effect of inoculum size on the time required for full growth.

occurred at all, whatever the inoculum size, such a difference would not be likely to be much exceeded under these conditions.

SUMMARY.

Proactinomycin in low concentrations lengthens the lag phase of the Oxford staphylococcus, and in certain media this increase in the lag becomes greater as the size of the inoculum is reduced. The prolonged lag obtained with small inocula is greatly decreased by filtrates from young cultures. It is considered that one of the primary effects of proactinomycin is an interference with the synthesis or use of diffusible substances produced by the cells and concerned with the ending of lag.

The length of the lag is increased considerably by raising the concentration of the antibiotic, but within a certain range the rate of division when logarithmic growth has set in is affected only to a minor extent. It appears as though a certain portion of the growth mechanism were relatively insensitive to proactinomycin.

When the concentration of proactinomycin is increased above a certain value its effect on the staphylococcus is of a different kind; after a period of incipient growth the cells then begin to die. In this respect there is a qualitative similarity between the effect of proactinomycin and that of penicillin.

The author is indebted to Miss R. Callow for expert technical assistance, and to Mrs. Philpot and Dr. Chain for a sample of purified proactinomycin.

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THE INFLUENCE OF GENETIC FACTORS ON THE TRANSMISSIBILITY OF FOWL SARCOMAS.

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ONE of the difficulties confronting the investigator of avian tumours is the elusive nature of the infective agent or virus which can be extracted from some spontaneous tumours of mesoblastic origin in the fowl. The best known examples of such tumours are the Rous sarcoma No. 1 and the Fujinami myxosarcoma, which is also infective for ducks. Other tumours which can be transmitted by cell-free virus and which differ histologically from the two mentioned above have been described by various authors, and the properties of such tumours have been critically reviewed by Foulds (1934).

It is at once apparent that there is difficulty in determining the presence or absence of an infective agent below a certain level of virulence. Some pathologists hold the view that because viruses can be demonstrated in some fowl sarcomas they must be postulated as the cause of all such tumours, and the generalization has even been extended to cover cancer in general. This is probably due to the universal method of classifying tumours on the basis of their histology.

In the case of most infective diseases classification on a histological basis would fail to differentiate their essential aetiology. Sometimes, as for example in tuberculosis, the histological appearance of the reacting tissue is sufficiently specific for diagnosis, but with other conditions the isolation of the infective organism or serological methods of detecting the specific antigen are necessary for precise diagnosis. With the infective tumours of fowls the position is further complicated by evidence that more than one type of histological reaction may be induced by the injection of extracts of blood or tissues of leukaemic fowls into the connective tissues of fresh hosts. Sarcoma, with or without an accompanying leukaemia, may follow such injections (Oberling and Guerin, 1933).

Though the origin of the viruses which can be extracted from the "filterable" fowl sarcomas and leucoses is not known, it is obvious that some birds constitute fresh sources of such agents, e.g. the original bearer of the Rous sarcoma No. 1. It therefore becomes a matter of importance to consider not only the source and nature of the inoculum when dealing with such tumour strains, but also the constitution of the fresh host which is to be inoculated.

Some breeds of fowl are apparently more prone to the spontaneous development of leucosis than others, and these should obviously be avoided in experiments in which search is being made for viruses in new tumour strains. In earlier experiments with chemically induced tumours in barred Plymouth Rocks of unknown pedigree one of us (P.R.P.), observed leucosis in several birds apparently unrelated to treatment. In an endeavour to avoid this possible source of confusion, fowls from the flock of Brown Leghorns maintained at the Institute of Animal Genetics, Edinburgh, have been used for such experiments since 1934.

It is the purpose of this communication to correlate, as far as possible, the results of inoculation of chemically induced fowl sarcomas with the genetic constitution of the hosts.

MATERIAL.

The flock of Brown Leghorns from which the experimental birds were obtained originally derives from a small foundation stock to which no new blood has been introduced since 1931. The birds are kept intensively, out of contact with the ground and other fowls. A population of approximately 500 breeding females is maintained, and all birds dying are examined by a competent poultry pathologist. Only one case of leukaemia has been diagnosed in the flock within the last 10 years.

In general the flock consists of lines developed for genetical research unconnected with cancer studies, and each inbred to varying degrees of intensity. Accurate records are kept of all stock bred. Chickens from four of these lines, " BP," " INT," " NM " and " LE," have been used in the experiments.

The tumour material used was derived from GRCH/13 and GRCH/15. The first was originally induced in Plymouth Rock fowls and later successfully transplanted into the Brown Leghorns. Since 1939 the dibenzanthracene sarcoma (GRCH/15), induced in a Brown Leghorn male (K.293), of the " BP " line has been used in extensive tests for the presence of a possible virus, all inoculations having been made into birds of the Edinburgh stock.

Three types of inoculum were employed in these investigations: (*a*) Cell inoculation; (*b*) cells submitted to various damaging procedures before inoculation; and (*c*) cell-free filtrates and desiccates. No positive results were obtained in group (*c*) and these data have been excluded; although the positives from group (*b*) were attributable to surviving tumour cells they also have been rejected, since all birds could not have been expected to receive adequate or even comparable amounts of infective material.

RESULTS.

Earlier work has shown that one of the lines of Brown Leghorns was significantly more resistant to inoculations of Rous No. 1 than others. Since the primary concern with the present material was to obtain birds in which chemically induced tumours took well, most of the chickens used were from the " BP " line, which has a history of being more susceptible to Rous No. 1 than the " NM " (resistant) line. Occasionally, however, birds from three other lines have been included, and although the numbers are small, they again indicate differences between the lines in respect of their resistance to tumour induction.

In Table I each of the three lines has been paired in turn with the " BP " lines, and the population tested for homogeneity of behaviour; the chi-squares show that the greater percentage of positives in the " INT " line was highly significant, that the " NM " line showed (as with the Rous No. 1 sarcoma) more resistance to the inoculations than the " BP," and that the third (" LE ") line was indistinguishable from the latter. While " BP " males have been recently introduced into the " LE " line, the others have been segregated practically from their initiation.

From an examination of the figures at Edinburgh it was noticed that an improvement in the number of takes appeared to have occurred in later years, and a test of homogeneity of the periods 1935–39 and 1940–44 resulted in the highly significant chi-square value of 16·7. Consultation between the authors disclosed that at the beginning of 1939 GRCH/15 had replaced GRCH/13 as the source of tumour material inoculated, and suggested that the former, originally induced in a " BP " Brown

TABLE I.—*Individual Tests of Homogeneity.* Susceptibility of "INT," "NM" and "LE" Lines Compared with "BP."*

Line.	% +.	+ Obs.	+ Exp.	− Obs.	− Exp.	Tot.	df.	χ^2	χ^2 Corrected for small nos.
"BP"	42·9	217	227	299	289	516			
"INT"	60·3	38	28	25	35	63			
"BP" + "INT"		255		324		579	1	7·22**	6·51**
"NM"	28·6	22	31	55	46	77	1	5·00*	4·48*
"LE"	38·2	13	14	21	20	34	1	0·18°	0·09°

(The expected "BP" ratio is shown for the 1st test only; the others can be deduced from the deviations in the "NM" and "LE" lines.)

* The statistic χ^2 provides a measure of the chances that the groups tested belong to the same population in respect of the character examined. In Table I the comparison of "BP" and "INT" lines gives a value of 7·22, a figure which is likely to occur less than 1 in 100 times with two samples of the same population; this is denoted by **. The χ^2 of 3·841 which corresponds to a 1 in 20 chance is the lowest value generally accepted as indicating a significant difference; it is also exceeded, in the comparison of "NM" with "BP," as is shown by the single * after 5·00 and 4·48. In Table II, where 6 groups are compared, the values of χ^2 required for significance are different, but *** after 36·81 shows that it is a highly significant figure, and that there are less than 1 in 1000 chances that the bi-monthly periods are behaving alike.

Leghorn, was in some way better adapted to this breed. (GRCH/13, as previously indicated, was originally induced in a Plymouth Rock fowl.) Transference of the 31 positive and 35 negative results obtained in 1939 from the first population to the second gave a positive-negative ratio for 1935–38 of 60 : 149, and for 1939–44, 157 : 150. This grouping increased the chi-square value to 25·7, thus emphasizing the difference still more.

A seasonal swing in the degree of susceptibility has been reported previously (Peacock, 1935). It was also detectable in the results of the present investigations, not only in the large "BP" group, but also in the smaller body of data obtained from the other lines when they were grouped together. The latter result is somewhat suspect, however, since in such a classification the lines may be compensating one another, and there are not sufficient data from any one of them for individual analysis.

The bi-monthly ratios for the "BP" line are shown in Table II. The total chi-square value calculated from them is so large that it leaves no doubt that their behaviour throughout the year is not uniform, while the period percentages taken in conjunction with the individual chi-squares are evidence in favour of a definite seasonal curve in the degree of susceptibility.

TABLE II.—*Seasonal Change in Susceptibility of the "BP" Line* (1935–1944).

	% +.	+ Obs.	+ Exp.	− Obs.	− Exp.	χ^2 for 217 : 299.
Feb.–Mar.	63·2	55	36·5	32	50·4	15·97
Apr.–May	38·7	29	31·5	46	43·5	0·34
June–July	29·5	33	47·1	79	64·9	7·28
Aug.–Sept.	26·8	15	23·6	41	32·4	5·41
Oct.–Nov.	28·3	44	48·4	71	66·6	0·69
Dec.–Jan.	57·7	41	29·9	30	41·1	7·12
		217		299		36·81***

DISCUSSION.

Analysis of the results has shown that the variable susceptibility of birds to transplanted tumours, which has been the common experience of investigators of fowl tumours, is not entirely capricious, but is governed in some way by genetic factors. Recently Cole (1941) has demonstrated genetic resistance in single comb White Leghorns to the transmissible Jungherr sarcoma. It was found possible to differentiate two lines by selective breeding, one characterized by a relatively high degree of resistance, and the other by a relatively low degree of resistance.

Although the birds used in the present investigations had not been selectively bred on the basis of tumour resistance, but were lines developed with regard to the fixation of specific characters relating to egg production, the results indicate that three genetic levels of resistance to tumour infection have been derived fortuitously in four lines of stock. The effect of the inbreeding involved has been to reduce genetic variability in the lines, not only in respect of the specific character selected for but also in associative characteristics. It may be that " BP " and " LE " represent a more heterozygous condition of a simple genetic situation involving resistance and susceptibility, and if so, such a conclusion would have far-reaching inferences. The results suggest that a more intensive approach should be made towards the definition of the heritable factors governing tumour transmission.

Resistance or susceptibility of an animal to the growth of the cells of another animal of the same or a different species may be partly explicable in terms of acquired immunity, that is to say, the development of specific antibodies to the inoculated cells, but may also be due to incompatibility of blood group, or to less specific causes of undetermined nature. Carr (1943) has shown that the Brown Leghorn fowls of the Edinburgh strain vary in susceptibility to the virus of the Rous Sarcoma No. 1. Some birds from the " NM " line may apparently resist infection altogether, or may grow a tumour which regresses.

Chemically induced tumours of fowls are of particular interest for comparative aetiological study, because the great majority of them can only be transmitted by cell inoculations. In this they resemble chemically induced and spontaneous mammalian tumours, and differ from the majority of transmissible fowl tumours of spontaneous origin. In the experiments under consideration two chemically induced fowl tumours, GRCH/13 and GRCH/15, were transmitted by cell inoculations. As no demonstration of a causal virus has ever been made of the GRCH tumour series, it is only possible to speak of susceptibility or resistance to the cells of these tumours (Peacock, 1933, 1935).

A point emerging from the investigations that should be stressed is that susceptibility to a particular tumour does not necessarily depend on close relationship between the host and the bearer of the original tumour ; thus, the sarcoma GRCH/13 which was induced in a Plymouth Rock hen failed to grow progressively in related Plymouth Rock chicks, but was maintained by serial transplantation through 22 passages in Brown Leghorns. Rous, in describing his sarcoma No. 1, recorded that the tumour could, at first, be transmitted only to blood relatives of the original tumour-bearing Plymouth Rock hen. In subsequent experiments, with another spontaneous fowl tumour (No. 18), which arose in a Brown Leghorn, transmission was most successful in Plymouth Rocks, as in the case of sarcoma No. 1 (Rous and Lange, 1913). Evidently it is a matter of chance whether a tumour occurs spontaneously in a bird with little inherent resistance to the growth of transplantable tumours.

This type of resistance or susceptibility to tumour transplantation may be peculiar and not necessarily related to resistance to filterable agents or to the spontaneous

growth of a tumour; it cannot be compared to the resistance of bacterial antigens. Similar results have already been described by Selbie (1936), who transplanted several well-known rat tumour strains in selectively bred rats. Though the nature of this genetic susceptibility remains obscure, it must be taken into account in critical experiments on the aetiology of induced transplantable fowl tumours.

A definite seasonal variation in tumour susceptibility is shown by the data collected over a period of 10 years. While the underlying significance of the phenomenon is not yet understood, it has been reported previously by Peacock (1935) and Murphy and Sturm (1941).

SUMMARY.

(1) Two strains of chemically induced sarcoma, one (GRCH/13) derived from a barred Plymouth Rock fowl, the other (GRCH/15), from a Brown Leghorn, have been maintained by serial passage in Brown Leghorn fowls of the Edinburgh flock.

(2) The results show consistent differences in susceptibility to both tumours by four inbred lines of fowls that had been selectively bred for purposes unrelated to cancer studies.

(3) The Brown Leghorn derived sarcoma was more easily grown in the Brown Leghorn stock than the GRCH/13 tumour derived from the barred Plymouth Rock, but the latter tumour could not be maintained in related birds.

(4) A definite seasonal variation in transmissibility of both tumours has been noted.

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